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CTFA
Cosmetic, Toiletry, and Fragrance Association

Pamela G. Bailey
President & CEO

May 4, 2006

Dr. Scott A. Masten
Director, Office of Chemical Nomination and Selection
NIEHS/NTP
111 T.W. Alexander Drive
P.O. Box 12233
Research Triangle Park, NC 27709

Rec'd 5/5/06

RE: Arbutin

Dear Dr. Masten:

The Cosmetic, Toiletry and Fragrance Association[1] (CTFA) appreciates the opportunity to provide additional information on substances that have been nominated for study by the NTP.

Attached, please find the following studies on Arbutin.

Acute Oral Toxicity Test. 1985
Acute Percutaneous Toxicity Test. 1986
Primary Skin Irritation Test. 1986
Patch Test of Arbutin in Humans. 1986.
Eye Irritation Test. 1986.
Skin Sensitization Test. 1986
In Vitro Percutaneous Absorption. 2003
Skin Metabolism After Repeated Topical Application of Arbutin in Human Volunteers. 2005
28-Day Oral Study. 1986
90-Day Percutaneous Study. 1986
Reverse Mutation Test. 1987
Chromosome Aberration Test. 1986
Percutaneous Carcinogenicity Study of Arbutin in Mice. 1996
One-Generation Reproduction Study by Subcutaneous Administration. 1986
Skin Photosensitization Test. 1986
Phototoxicity Test. 1986

I hope you find this information helpful.

Sincerely,

John E. Bailey, Ph.D.
Executive Vice President - Science

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[1]Based in Washington, D.C., CTFA is the trade association representing the cosmetic, toiletry, and fragrance industry in the United States and globally. Founded in 1894, CTFA has a membership of nearly 600 companies including manufacturers, distributors, and suppliers for the vast majority of finished personal care products marketed in the United States.

Acute Oral Toxicity Test of Arbutin in Mice and Rats

Acute Oral Toxicity Test of Arbutin in Mice and Rats

Koya Shiratori, Hiroaki Eiro,
Hiroko Matsumoto, Shin-ichi Hirama,
Kumi Yoshihara, Masashi Yanagi
Yoshikuni Wakisaka

1. Summary

The acute oral toxicity of Arbutin was examined in mice and rats.

(1) The LD_{50} was 10664 and 9804 mg/kg in male and female mice, and 8715 and 9321 mg/kg in male and female rats. Rats generally had lower values than mice.

(2) Mice and rats displayed almost identical signs of toxicity, and there was no difference in toxic signs between sexes. The degree of toxic signs was more severe in rats than in mice. Toxic signs occurred immediately after administration at 4919 mg/kg or higher dose levels in both mice and rats. Toxic signs included reduced spontaneous motor activity, ptosis, and lying prone. Shivering and clonicity were also observed at 9641 mg/kg and higher. Signs at these dose levels disappeared on the day following dosing. Signs of toxicity were not observed at 3513 mg/kg or less.

(3) Mortality did not occur until dose levels reached 4919 mg/kg in mice and at 6886 mg/kg in rats.

(4) Body weight was reduced in both sexes until the first 3 or 4 days after administration in mice given 13496 mg/kg. No other remarkable observations were made for body weight in mice. Body weight increased normally in both male and female rats.

(5) Distention of the digestive tract was observed at necropsy in mice and rats that died during the test period. The cecum retained a large volume of contents, but there were no remarkable findings in other organs. In surviving mice, irregular yellowish spots were sporadically observed in livers of males given 6886 mg/kg and females given 4919 mg/kg. Scar tissue was seen in the liver of rats given 9641 mg/kg.

(6) There were no remarkable findings by light microscopy in animals that died. In surviving mice, focal or zonal hepatic necrosis corresponded to gross lesions observed at necropsy. Increased supcapsular connective tissue corresponded to gross liver lesions in rats.

2. Introduction

This study evaluated the acute oral toxicity of Arbutin in mice and rats.

The study was conducted between December 3 and 17, 1985.

3. Materials and Methods

3.1 Test substance

Arbutin (Lot a) was used as the test substance.

3.2 Animals

Male and female SPF ICR mice (Crj:CD-1) and male and female SPF SD rats (Crj:CD), were purchased from Charles River Japan Inc. at 4 weeks of age. After an acclimation period of 6 days, animals that appeared normal were divided into groups of 5 each for the study. Body weights of the dosing day were in the range of 22.1 to 30.4 g for male mice, 17.6 to 24.0 for female mice, 76.2 to 104.2 for male rats, and 64.8 to 95.4 g for female rats respectively.

3.3 Housing environment

The animals were housed throughout the acclimations and the test periods in a barrier facility. Temperature and humidity of the animal quarters were maintained at 23 ± 2ºC and 55 ± 5% RH, respectively, with an air exchange frequency of 32 times/hour and a light cycle of 12 hours. Mice were housed in plastic cages with bedding chips (215 x 130 x 320 mm: Clea Japan Inc., Tokyo, Japan). Rats were kept in suspended wire mesh metal cages (300 x 200 x 400 mm: Clea Japan Inc., Tokyo, Japan). Animals were housed 5 per cage and fed laboratory chow (radiation-sterilized, NMFR: Oriental Yeast Co., Ltd.) and tap water (ultraviolet ray and microfilter-treated) *ad libitum*.

3.4 Dosing

Both mice and rats received test substance by gavage after 16 hours of food deprivation. Distilled water was used as the vehicle. Solubility of the test substance in water increases with temperature. Solubility at 37ºC is 25% (w/w), and therefore a 25% aqueous solution (specific gravity: 1.0797), heated to 37ºC, was used for oral dosing. Each dosing group consisted of 5 animals. Eight dose levels, from 1792 to 18895 mg/kg, were established in a ratio of 1.4 to 1. Dose levels were varied by adjusting the dose volume.

3.5 Observations

Clinical signs were recorded for 14 days. Body weight was measured on day 1 to 5, 7, 8, 11, and 15. LD_{50} was calculated by van der Waerden's method on the basis of cumulative mortality until 14 days after dosing. Animals that died during the observation period were immediately subjected to necropsy, and survivors were sacrificed with chloroform at the end of observation period and subjected to necropsy. Selected animals were histopathologically examined.

4. Results

4.1 Mice

1) LD_{50}

The oral LD_{50} was 10664 mg/kg in males and 9804 mg/kg in females (Table 1)

2) Clinical signs

Decrease in locomotor activity, closed eyelids, ataxic gait, and prone position appeared immediately after dosing in both sexes at 4919 mg/kg and higher. These toxic signs were observed early in high dose groups. Tremor and clonic convulsion appeared in addition to these signs in animals given 9641 mg/kg or higher.

Clonic convulsion was severe in dying animals. Lateral turning was also sometimes observed in moribund animals. Additional observations included salivation and periproctal soiled. Succumbing animals died within 2 to 24 hours. Deaths occurred beginning at 4919 mg/kg in males and at 6886 mg/kg in females.

Toxic signs disappeared on the following day in survivors. Recovery was uneventful. No remarkable signs of toxicity were observed at 3513 mg/kg or less.

3) Body weight

Body weight was reduced in both sexes at 13496 mg/kg until day 3 or 4, and then increased normally thereafter. Weight gain was normal at lower dose levels (Table 2, 3).

4) Necropsy findings

The findings were identical in both males and females.

Distention of the stomach, small intestine, and cecum were observed in animals that succumbed. Stomach contents were viscous with test substance. The cecum contained a large volume of muddy or aqueous contents. These findings were remarkable in those animals given 18895 mg/kg that died within several hours of dosing. Yellowish lobular structures in the liver, and discoloration in kidneys and spleen were observed in some of the animals dying on day 1 after administration of the test substance (groups given 4919 to 13496 mg/kg).

Irregular-shaped yellowish spots the size of a grain of rice or red bean were sporadically observed in livers of surviving males given 6886 mg/kg and females given 4919 mg/kg (Photo. 1). In addition, two pinpoint-sized ulcers were found in the proventriculus of a male animal given 9641 mg/kg. No other remarkable observations were made for other organs.

5) Histopathology

No remarkable changes were observed in animals that died during the observation period.

In survivors, focal or zonal [liver] necrosis was observed in some animals given 4919 mg/kg or higher doses. Focal necrosis was typically acidophilic, but occasionally basophilic due to the deposition of calcium. Slight to moderate infiltration of polymorphonuclear leukocytes and monocytes were observed around necrotic areas. Multinuclear giant cells appeared prominently. Increases of connective tissue were observed around focal necroses and within zones of necrotic liver cells (Photo. 2). No remarkable observations were made for other organs.

4.2 Rats

1) LD_{50}

The oral LD_{50} was 8715 mg/kg in males and 9321 mg/kg in females (Table 1).

2) Clinical signs

Decrease in locomotor activity, closed eyelids, ataxic gait, and prone position appeared immediately after dosing in both sexes beginning at a dose level of 4919 mg/kg. The degree of these clinical signs showed clear dose-dependence. Severity of these clinical signs was milder than in mice. Four hours after administration, Tremor and clonic convulsion were also observed in animals given 9641 mg/kg or more, but the severity was less than in mice. Salivation and periproctal soiled was observed.

Succumbing animals died within 5 to 24 hours.

In survivors, all the symptoms disappeared on the day following the dosing and they recovered normally. Almost no clinical signs were observed in groups given 3513 mg/kg or less.

3) Body weight

Body weight gain in each group appeared normal throughout the observation period (Table 4, 5).

4) Necropsy findings

Findings were identical between sexes.

Distention of the stomach, small and large intestines were observed in dying animals. Stomachs contents were clear and viscous. Cecum contained a large amount of gray-green muddy or aqueous contents. No remarkable changes were observed in other organs.

In survivors, sporadic linear or irregularly shaped grayish white cicatricial changes were observed on the diaphragmatic and visceral surfaces of the left lobe of the liver in one male and two females given 9641 mg/kg (Photo. 3). No abnormalities were observed with other organs.

5) Histopathology

No remarkable changes were observed in animals that died during the observation period.

In survivors, a slight increase in connective tissue was observed subcapsularly in the liver.

These changes were found at necropsy in the groups given 9641 mg/kg (Photo. 4). No remarkable changes were observed in other organs.

5. Conclusion

The oral LD_{50} of Arbutin (10664 mg/kg in male mice, 9804 mg/kg in female mice, 8715 mg/kg in male rats, and 9321 mg/kg in female rats) indicates a low order of toxicity. Few toxic signs were observed in either species at dose levels up to 3513 mg/kg. There were no deaths in mice given less than 3513 mg/kg or in rats given less than 4919 mg/kg. In survivors, liver changes associated with the test substance were observed at 4919 mg/kg in mice and 9641 mg/kg in rats. Since these changes were not observed with groups at the lower dose levels, it is concluded that oral toxicity of Arbutin is of a low order.

Acute Percutaneous Toxicity Test of Arbutin in Mice and Rats

Acute Percutaneous Toxicity Test of Arbutin in Mice and Rats

Koya Shiratori, Hiroaki Eiro,
Hiroko Matsumoto, Shin-ichi Hirama,
Kumi Yoshihara, Masashi Yanagi,
Yoshikuni Wakisaka

1. Introduction

The following study evaluated the acute percutaneous toxicity of Arbutin in mice and rats.

The study was conducted from January 30 to February 13, 1986 (rats) and from March 20 to April 3, 1986 (mice).

2. Materials and Methods

2.1 Test substance

Arbutin (Lot a) was used as the test substance.

2.2 Animals

Male and female SPF ICR mice (Crj:CD-1), and male and female SPF SD rats (Crj:CD) were purchased at 4 weeks of age from Charles River Japan Inc. After an acclimation period of 6 days, animals that appeared normal were divided into groups of 10 each for the study.

2.3 Housing environment

The animals were housed throughout the acclimation and test periods in a barrier facility. Temperature and humidity of the animal quarters were maintained at 23 ± 2ºC and 55 ± 5% relative humidity, respectively, with an air exchange frequency of 32 times/hour and a light cycle of 12 hours. Mice were housed in plastic cages with bedding chips (125 x 200 x 110 mm: Clea Japan Inc., Tokyo, Japan). Rats were housed in suspended wire-mesh metal cages (300 x 200 x 400 mm: Clea Japan Inc., Tokyo, Japan). Animals were fed with laboratory chow (radiation-sterilized, NMFR: Oriental Yeast Co., Ltd.) and tap water (ultraviolet ray and microfilter-treated) *ad libitum*. Bedding chips were removed for 24 hours after administration of the test substance in order to prevent contamination of bedding with test substance.

2.4 Dosing

For both rats and mice, the test substance was applied evenly on the back after clipping the fur. Dose volume was 3 ml/kg, the maximum technically feasible. The vehicle was 50% aqueous ethanol solution. Solubility of the test substance in the vehicle is 30% (w/w) at 37ºC. The concentration of Arbutin in the dosing solution was therefore set at 30%, providing for a dose of 928 mg/kg. The specific gravity of the dosing solution was 1.0309.

2.5 Observations

For mice, clinical signs were recorded for 14 days except for holiday, and body weight was measured on day 1 to 3, 5 to 8, 12 and 15. For rats, clinical signs were recorded for 14 days, and body weight was measured on day 1 to 3, 5 to 8, and 15. Since no animal died during the observation period, all animals were sacrificed with chloroform at the end the observation period and subjected to necropsy.

3. Results

Table 1 LD_{50} of Arbutin

Animal species	Sex	Dose (mg/kg)	Mortality	LD_{50} (mg/kg)
Mouse	Male	928	0 / 10	> 928
	Female	928	0 / 10	
Rat	Male	928	0 / 10	> 928
	Female	928	0 / 10	

3.1 LD_{50}, clinical signs, and body weight

No animal died in any group during the observation period and the LD_{50} was considered to be greater than 928 mg/kg (Table 1). No abnormalities were seen in clinical signs. Body weight gain was normal throughout the observation period (Table 2, 3).

3.2 Necropsy findings

There were no remarkable findings at necropsy for either mice or rats.

4. Conclusion

The acute percutaneous toxicity of Arbutin was evaluated in mice and rats.

The LD_{50} was greater than 928 mg/kg. The maximum technically applicable dose was 928 mg/kg. There were no remarkable findings in clinical signs, body weight or necropsy. It is concluded that the percutaneous toxicity of the test substance is of a low order.

Table 2 Body weight of Mice

Dose (mg/kg)	928 Male	928 Female
No.of animals	10	10
Days		
1	28.4 ± 1.1	24.1 ± 1.5
2	28.5 ± 1.6	24.3 ± 1.4
3	29.2 ± 1.4	24.4 ± 1.4
5	30.0 ± 1.6	24.8 ± 1.5
6	30.7 ± 1.4	24.6 ± 1.7
7	31.3 ± 1.5	25.3 ± 1.4
8	31.3 ± 1.8	25.1 ± 1.2
12	29.7 ± 2.0	23.5 ± 1.8
15	32.6 ± 2.7	26.6 ± 1.8

Mean±S.D. Unit: g

Table 3 Body weight of Rats

Dose(mg/kg)	928 Male	928 Female
No.of animals	10	10
Days		
1	146.2 ± 4.4	109.3 ± 4.6
2	152.6 ± 5.4	112.0 ± 6.7
3	166.9 ± 4.5	120.7 ± 6.4
5	185.3 ± 6.1	131.7 ± 7.5
6	194.4 ± 6.2	132.7 ± 8.2
7	202.6 ± 7.3	136.0 ± 8.0
8	207.2 ± 7.9	140.6 ± 8.3
15	274.6 ± 9.8	173.6 ± 31.9

Mean±S.D. Unit: g

Primary Skin Irritation Test of Arbutin in Rabbits

Primary Skin Irritation Test of Arbutin in Rabbits

Junko Tanaka, Masato Kuramoto,
Hiroshi Tanaka, and Toshiaki Kobayashi

1. Introduction

This study evaluated the primary skin irritation potential of Arbutin in rabbits.
The study was conducted from April 1 to April 4, 1986.

2. Materials and Methods

2.1 Test substance

Arbutin (Lot a, Nippon Fine Chemical Co., Ltd.) was used as the test substance.

Since the original substance is a crystalline powder, it was dissolved in distilled water to 10% concentration for application.

2.2 Animals

Japanese white male rabbits (1.8 to 2.0 kg) were purchased. After a 2-week acclimatization, animals weighed 2.3 to 2.8 kg, and those appeared normal were selected for the study.

2.3 Environmental conditions and housing

Animals were housed individually in aluminum rabbit bracket cages (350 x 500 x 350 mm, Clea Japan Inc., Tokyo, Japan). They were fed laboratory chow (RC-4: Oriental Yeast Co., Ltd.) and tap water *ad libitum*. Animal quarters were automatically controlled to 23 ± 2ºC and 55 ± 5% relative humidity.

2.4 Test methods

The test was conducted according to the method of Draize[1)]. Six rabbits were individually placed in restraint devices (HZL type) and fur was clipped from the dorsal skin. This application area was divided into two sections. The skin of the first section was left intact and that of the other section was abraded in the area to be covered by the patch by scratching a “#” (sharp) symbol with a hypodermic needle. The abrasion remained within the stratum corneum of the skin without reaching the corium, and was not deep enough to cause bleeding.

Arbutin (0.3 ml of a 10% solution) was applied to the skin using a patch-test plaster with a lint pad diameter of 2.5 cm (Torii Pharmaceutical Co., Ltd.).

Animals were restrained for 24 hours. Care was taken to avoid stressing the animals as much as possible during restraint. After 24 hours, the plaster was removed and the skin reaction (erythema and edema) was scored according to the criteria given below. Rabbits were then individually housed in aluminum rabbit bracket cages. The skin reaction was again evaluated after 72 hours.

To calculate the primary skin irritation index, erythema and edema scores on the intact and abraded skin at 24 and 72 hours were separately subtotaled, averaged across the two observation intervals, and the averages were then summed across intact/abraded skin sections and rabbits.

Skin reaction was evaluated under ambient laboratory illumination according to the following criteria:

Criteria

a) List of rating scores

(1) Erythema and Eschar Formation

Criteria	Score
No erythema perceptible	0
Slight erythema perceptible	1
Well defined erythema	2
Severe erythema	3
Severe erythema to slight eschar formation	4

(2) Edema Formation

Criteria	Score
No edema perceptible	0
Very slight edema (barely perceptible)	1
Slight edema	2
Moderate edema (area raised approx. 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure)	4

b) Evaluation of the Primary Irritation Index

Score	Evaluation
0 to 2.0	Almost no irritation
2.1 to 5.0	Moderate irritation
5.1 to 8.0	Severe irritation

3. Results

Primary skin irritation of a 10% solution of Arbutin was evaluated in rabbits. Slight erythema was observed in the intact and abraded skin of one rabbit at 24 and 72 hours. No edema was observed for any the rabbit during the test period. (Table 1)

Table 1 Results of the primary skin irritation test (rabbit)

Test substance: Arbutin

Dose: 0.3 ml Concentration: 10% Solvent: Distilled water

Test animal No.	Skin	Erythema		Edema		Average
		24h	72h	24h	72h	
1	Intact skin	0	0	0	0	0.0
	Abraded skin	0	0	0	0	0.0
2	Intact skin	0	0	0	0	0.0
	Abraded skin	0	0	0	0	0.0
3	Intact skin	0	0	0	0	0.0
	Abraded skin	0	0	0	0	0.0
4	Intact skin	0	0	0	0	0.0
	Abraded skin	0	0	0	0	0.0
5	Intact skin	1	1	0	0	0.5
	Abraded skin	1	1	0	0	0.5
6	Intact skin	0	0	0	0	0.0
	Abraded skin	0	0	0	0	0.0
					Total	1.0
Primary skin irritation index 1.0/6 ≒ 0.2						

4. Conclusion

The primary skin irritation of Arbutin was evaluated in rabbits. The primary skin irritation index for a 10% solution of Arbutin was 0.2, which is considered as having almost no irritation.

In conclusion, Arbutin has little primary skin irritation potential.

5. Reference

1) Draize JH (1959) Dermal toxicity. Appraisal of the safety of chemicals in foods, drugs and cosmetics. Association of Food and Drug Officials of the United States, Texas State Department of Health. Texas: Austin.

Patch test of Arbutin in humans

Hiroko Tamura, Rie Ikeda, Hiroshi Tanaka and Toshiaki Kobayashi

1. Objective

The objective of the present test was to investigate the irritation potential of Arbutin on human skin.

2. Summary

Application	: April 1, 1986
48-hour reading	: April 3, 1986
72-hour reading	: April 4, 1986

3. Materials and Methods

3.1 Test material

Arbutin (Nihon Seika Inc., Lot No. a) was used for the test substance.

Arbutin was dissolved in distilled water to 10% concentration (Hereinafter referred to as the 10% Arbutin solution).

3.2 Subjects and test site

Subjects consisted of 43 healthy male individuals ranging in age from 25 to 47 years (mean age: 35 years). The test area was the back of the subject.

3.3 Test methods

A 48-hour closed patch test was performed using plasters for human patch test (16 mm in diameter; Torii Pharmaceuticals Co.). For each subject, 0.05 ml of 10% Arbutin solution was placed on a piece of lint, which was then attached to the back of the subject. The application site was then immobilized using Nichiban Keepsilk plasters. After 48 hours, the plasters were removed. The first reading was performed 30 minutes after the removal (48-hour reading), and the severity of skin reactions was assessed according to criteria shown in Table 1. A second reading was performed 24 hours later (72-hour reading).

Table. 1 Reading criteria

Severity of skin reactions	Reading
No reaction	(-) negative
Mild erythema	(±) pseudo-positive
Erythema	(+) positive, weak
Erythema + edema	(++) positive, medium
Erythema + edema + papules, serous papules or small vesicles	(+++) positive, strong
Large vesicles	(++++) positive, strongest

4. Results

The irritation potential of Arbutin was investigated on the backs of 43 healthy men in a 48-hour closed patch test.

No positive reactions were seen at 48 or 72 hours after application of 10% Arbutin solution, and the positive rate was 0% (Tables 2 and 3).

5. Conclusions

The irritation potential of Arbutin was investigated by conducting a 48-hour closed patch test on the backs of 43 healthy volunteers. Readings performed at 48 and 72 hours after application of 10% Arbutin solution revealed no positive skin reactions. Therefore, the irritation potential of Arbutin was concluded to be low.

Table. 2 Results of 48-hour closed patch test for Arbutin in humans (summary)

Test material	Number of subjects	Reading time (hrs.)	Positive reactions				Pseudo-positive (±)	Negative (-)	Positive rate* (%)
			(++++)	(+++)	(++)	(+)			
10% Arbutin solution	43	48	0	0	0	0	4	39	0
		72	0	0	0	0	0	43	0

*: Positive rate = (number of positive cases/total number of subjects) × 100

Table 3 Results of 48-hour closed patch test for Arbutin in humans (part 1)

No.	Subjedt code	Judge time	Judgement	No.	Subject code	Judge time	Judgement
			Test substance A				Test substance A
1	L-9011	48	–	13	L-1003	48	–
		72	–			72	–
2	L-9010	48	–	14	L-1061	48	–
		72	–			72	–
3	L-9004	48	–	15	L-1033	48	±
		72	–			72	–
4	L-9003	48	–	16	L-9005	48	–
		72	–			72	–
5	L-9007	48	–	17	L-9008	48	–
		72	–			72	–
6	L-9014	48	–	18	L-9006	48	–
		72	–			72	–
7	L-1071	48	–	19	L-2045	48	–
		72	–			72	–
8	L-1039	48	±	20	L-2013	48	–
		72	–			72	–
9	L-1020	48	–	21	L-2047	48	–
		72	–			72	–
10	L-1040	48	–	22	L-2028	48	–
		72	–			72	–
11	L-1042	48	–	23	L-2039	48	–
		72	–			72	–
12	L-1070	48	–	24	L-2034	48	–
		72	–			72	–

Test substance A: 10% Arbutin solution

Table 3 Results of 48-hour closed patch test for Arbutin in humans (part 2)

No.	Subjedt code	Judge time	Judgement	No.	Subject code	Judge time	Judgement
			Test substance A				Test substance A
25	L-2001	48	–	37	L-3032	48	–
		72	–			72	–
26	L-2006	48	–	38	L-3006	48	–
		72	–			72	–
27	L-2009	48	–	39	L-3016	48	–
		72	–			72	–
28	L-2003	48	–	40	L-3007	48	–
		72	–			72	–
29	L-2048	48	–	41	L-3005	48	–
		72	–			72	–
30	L-2040	48	–	42	L-3030	48	–
		72	–			72	–
31	L-2020	48	–	43	L-3010	48	–
		72	–			72	–
32	L-2022	48	±				
		72	–				
33	L-2024	48	±				
		72	–				
34	L-2049	48	–				
		72	–				
35	L-2036	48	–				
		72	–				
36	L-3008	48	–				
		72	–				

Test substance A: 10% Arbutin solution

Eye Irritation Test of Arbutin in Rabbits

Eye Irritation Test of Arbutin in Rabbits

Junko Tanaka, Masato Kuramoto,
Hiroshi Tanaka, and Toshiaki Kobayashi

1. Introduction

This study evaluated eye irritation potential of Arbutin in rabbits.

The study was conducted from March 25 to April 1, 1986.

2. Materials and Methods

2.1 Test substance

Arbutin (Lot a, Nippon Fine Chemical Co., Ltd.) was used as the test substance.

Since the original substance is a crystalline powder, it was dissolved in distilled water to a 10% concentration for application.

2.2 Animals

Japanese white male rabbits (1.8 to 2.0 kg) were purchased. After a 2-week acclimatization, animals weighed 2.3 to 3.5 kg, and those appeared normal were selected for the study. An aliquot (0.1 ml) of a 2% fluorescein sodium solution was instilled to visualize any corneal damage, and animals displaying eye abnormalities were rejected.

2.3 Environmental conditions and housing

Animals were housed individually in aluminum rabbit bracket cages (350 x 500 x 350 mm: Clea Japan Inc., Tokyo, Japan). They were fed laboratory chow (RC-4: Oriental Yeast Co., Ltd.) and tap water *ad libitum*. Animal quarters were automatically controlled to 23 ± 2ºC and 55 ± 5% relative humidity.

2.4 Test method

An aliquot (0.1 ml) of a 10% Arbutin solution was instilled to the right eyes of three rabbits. No irrigation was given. Untreated left eyes served as control.

Collars were placed on animals immediately after instillation of the test substance solution.

Ocular reaction was observed for one week according to the Draize method. Evaluations were performed according to the following table of scores.

Table of scores

(1) Cornea

(A_1) Opacity: Degree of density (area most dense taken for reading)

Transparent	0
Diffuse or scattered areas of opacity Details of iris clearly visible	1
Easily discernible translucent areas Details of iris slightly obscured	2
Opalescent areas. No details of iris visible Size of pupil barely discernible	3
Opaque. Iris invisible	4

(B_1) Area of cornea involved

0	0
> 0 to 1/4	1
1/4 to 1/2	2
1/2 to 3/4	3
> 3/4	4

Sum: (A_1) x (B_1) x 5, maximum theoretical value = 80

(2) Iris

(A_2) Morbidity value

Normal	0
Rugae deepened, congestion, swelling, circumcorneal injection. Iris still reacting to light	1
No reaction to light, hemorrhage, gross destruction	2

Sum: (A_2) x 5, maximum theoretical value = 10

(3) Conjunctiva

(A_3) Redness

Normal blood vessels	0
Vessels definitely congested above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3

(B_3) Chemosis

No chemosis	0
Any swelling above normal	1
Obvious swelling with partial erosion of the lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4

(C_3) Discharge

No discharge	0
Any amount different from normal	1
Discharge with moistening of the lids and hair just adjacent to the lids	2
Discharge with moistening of the lids and considerable area around the eye	3

Sum: $[(A_3)+(B_3)+(C_3)] \times 2$, maximum theoretical value = 20

3. Results

Eye irritation of a 10% solution of Arbutin was evaluated in rabbits without irrigation. No reaction was observed in the cornea, iris or conjunctiva during the test period (Table 1, Figure 1).

4. Conclusion

Eye irritation potential of Arbutin was evaluated in rabbits. No reaction was observed in the cornea, iris or conjunctiva after instillation of a 10% Arbutin solution without irrigation.

In conclusion, Arbutin has little eye irritation potential.

Table 1 Eye irritation test in rabbits

Rabbit No.	
	No. 1
	No. 2
	No. 3

Test substance: Arbutin (no irrigation)

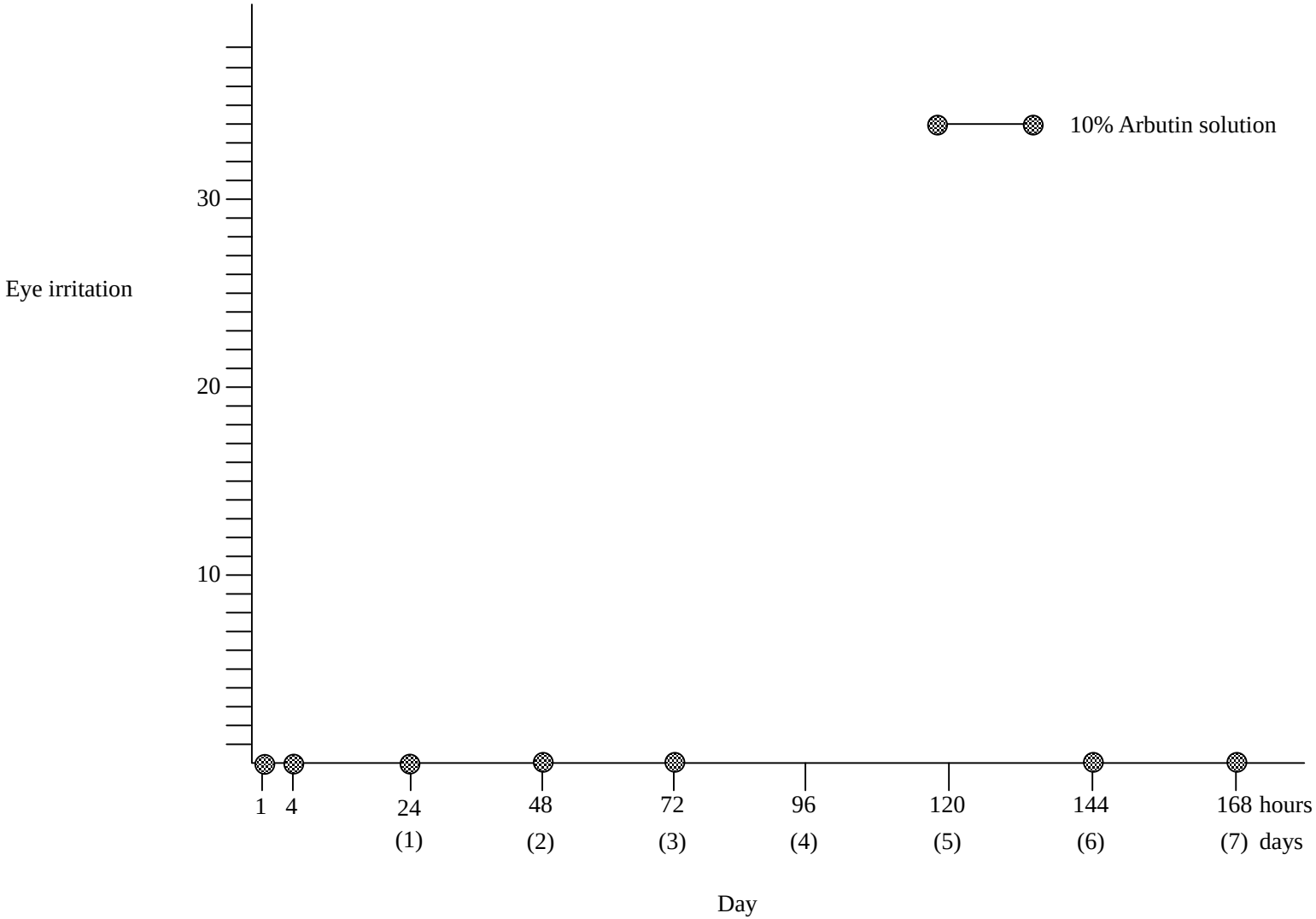
Dose: 0.1 ml Concentration: 10% Solvent: Distilled water

Elapsed time and day	Male No. 1										Male No. 2										Male No. 3										Average rating
	Cornea			Iris		Conjunctiva				Total rating	Cornea			Iris		Conjunctiva				Total rating	Cornea			Iris		Conjunctiva				Total rating	
	Opa	Ar	Total	Morbid value	Total	Red	Che	Dis	Total		Opa	Ar	Total	Morbid value	Total	Red	Che	Dis	Total		Opa	Ar	Total	Morbid value	Total	Red	Che	Dis	Total		
	a	b	a x b x 5	a	a x 5	a	b	c	(a+b+c) x 2		a	b	a x b x 5	a	a x 5	a	b	c	(a+b+c) x 2		a	b	a x b x 5	a	a x 5	a	b	c	(a+b+c) x 2		
1 hour	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4 hour	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24 hour	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2 days	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3 days	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4 days																															
5 days																															
6 days	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7 days	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Opa = Opacity Ar = Area Red = Redness Che = Chemosis Dis = Discharge

Fig. 1 Change in eye irritation by days (no irrigation)

Eye irritation = Average rating for the cornea, iris and conjunctiva



Skin Sensitization Test of Arbutin in Guinea Pigs

Skin Sensitization Test of Arbutin in Guinea Pigs

Hideyuki Ichikawa, Yoshio Katsumura,
Shinobu Ishii, and Toshiaki Kobayashi

1. Introduction

This study evaluated the skin sensitizing potential of Arbutin in guinea pigs.
The study was conducted from May 14 to June 6, 1986.

2. Materials and Methods

2.1 Test substance

Arbutin (Lot a, Nippon Fine Chemical Co., Ltd.) was used as the test substance. The comparative substance was hydroquinone (Lot 110913, Mitsui Petrochemical Industries, Ltd.), for which clinical reports on contact dermatitis[1) 2)] and reports of animal tests[3)] are available.

2,4-Dinitrochlorobenzene (DNCB, Lot EPR5822, Wako Pure Chemical Industries, Ltd.) was used as the positive control substance.

2.2 Animals

Hartley strain female albino guinea pigs weighing about 350 g were purchased. After an acclimation period of one week, guinea pigs weighing between 380 and 450 g that appeared normal were used.

2.3 Environmental conditions

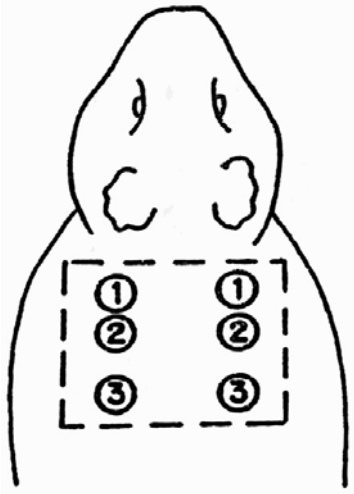
Animals were housed individually in aluminum guinea pig bracket cages (260 x 170 x 380 mm: CLEA Japan Inc., Tokyo, Japan) during the acclimatization and the test periods. They were fed laboratory chow (RC-4: Oriental Yeast Co., Ltd.) and tap water *ad libitum*. Animal quarters were automatically controlled to 23 ± 2ºC and 55 ± 5% relative humidity.

2.4 Sensitization test method

The Guinea Pig Maximization test method[4)] was used.

2.4.1 Sensitization induction

Thirty-five guinea pigs were used; ten were treated with Arbutin, ten with hydroquinone, five with DNCB, and ten with distilled water (control group). Fur of the shoulder area was clipped with electric clippers, and then shaved with an electric shaver. The following operations were performed on 3 × 4 cm shaved areas of skin:



1) Freund's complete adjuvant (Difco Laboratories) and equal volume of distilled (Otsuka Pharmaceutical Co., Ltd.) were emulsified (water-in-oil type emulsification). This emulsion (0.1 mL) was injected intradermally at two points labeled ① in the figure.
2) Arbutin was dissolved in distilled water at a concentration of 10%. (An aqueous solution was used as the vehicle, because a 50%, v/v, aqueous ethanol solution causes tissue necrosis.) Arbutin solution (0.1 mL) or 0.1 mL of a 5% solution of hydroquinone in distilled water was injected intradermally at two points labeled ② in the figure. DNCB is oil-soluble; therefore, it was dissolved in liquid paraffin (Lot JT-C-AE-2, Esso and Standard Oil Company) to a concentration of 0.1%. This solution (0.1 mL) was injected intradermally at two points labeled ② in the figure. Distilled water was injected into the control animals in place of the test or positive control substance solutions.
3) Arbutin in distilled water (20%) and equal volume of Freund's complete adjuvant were emulsified (water-in-oil type emulsion) to a final Arbutin concentration of 10%. Hydroquinone was dissolved in distilled water to its limit of solubility (10%) determined in preliminary testing and emulsified with an equal volume of Freund's complete adjuvant (water-in-oil type emulsion) to a final hydroquinone concentration of 5%. These emulsions (0.1 mL) were injected intradermally at two points labeled ③ in the figure for the respective sensitization groups. DNCB was dissolved in Freund's complete adjuvant to a concentration of 0.2%, and then emulsified (water-in-oil type emulsion) with an equal volume of distilled water to attain a final DNCB concentration of 0.1%. This emulsion (0.1 mL) was injected intradermally to the two points in section ③ in the figure.
4) On Day 7 after induction of sensitization, fur of the shoulder area was again shaved with an electric shaver and the skin was treated with 50 mg of 10% sodium Lauryl sulfate in petrolatum to accelerate percutaneous absorption.
5) Twenty-four hours after surfactant application, 0.2 mL of each test substance was absorbed into a 2 x 4 cm piece of filter paper, which was applied as an occlusive dressing for forty-eight hours. Distilled water was absorbed to the filter paper applied to control

animals.

2.4.2 Challenge exposure

Challenge exposure was carried out on Day 21 after induction of sensitization. Fur was removed from the flank by clipping and shaving as before. Ten microliters of test sample were applied directly to an approx. 1-cm^2 area of the flank skin. Arbutin at concentrations of 10, 3 and 1% in 50%, v/v, aqueous ethanol was topically applied to the flanks of animals that were sensitized with Arbutin. Hydroquinone at concentrations of 10, 3 and 1% in 50%, v/v, aqueous ethanol was topically applied to flanks of animals that were sensitized with hydroquinone. DNCB at concentrations of 0.1 and 0.01% in acetone were topically applied to flanks of animals that were sensitized with DNCB. For the animals that were sensitized with distilled water (control group), the test substance was similarly applied to an area of the flank skin.

The skin reaction with respect to erythema and edema was evaluated according to the scoring criteria below at 24 and 48 hours after the challenge exposure.

Criteria

(1) Erythema formation

Criteria	Score
No erythema	0
Very slight erythema	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema with eschar formation	4

(2) Edema formation

Criteria	Score
No edema	0
Slight edema	1
Moderate edema	2
Severe edema	3

3. Results

Table 1 displays the results of evaluation of the skin sensitization potential of Arbutin performed according to the guinea pig maximization test method. Table 2 shows the results for hydroquinone used as the comparative substance. Table 3 shows the results for DNCB used as the positive control substance.

No positive reactions were observed at any reading time in animals in either the treated or control groups challenged with 10, 3 and 1% Arbutin in 50%, v/v, aqueous ethanol solution.

Positive reactions to the 10% hydroquinone challenge concentration were observed at 24 hours in nine of ten animals sensitized with hydroquinone. At 48 hours, positive reactions were observed in all ten guinea pigs. One had severe erythema with necrosis, six had moderate to severe erythema, two had well-defined erythema, and one had very slight erythema. Of animals with positive reactions, two had edema, one severe and the other slight. At the challenge concentration of 3%, positive reactions were observed in eight of ten animals at 24 hours. A 48 hours, all ten displayed positive reactions: one very severe erythema with necrosis, five moderate to severe erythema, two well-defined erythema and two very slight erythema. One of the animals with positive reactions had edema. At a challenge concentration of 1%, six guinea pigs were observed to have positive reactions at 24 hours. At 48 hours, nine guinea pigs were observed to have positive reaction, two of which had moderate to severe erythema, four well-defined erythema and three very slight erythema. Conversely, no positive reactions were observed in control group at any challenge concentration.

In the group sensitized with DNCB as the positive control substance, five of five were observed to have moderate to severe erythema to the challenge concentration of 0.1% at 24 and 48 hours. Of these animals, two and one were observed to have slight edema after twenty-four and forty-eight hours, respectively. At the challenge concentration of 0.01%, three guinea pigs were observed to have slight edema at 24 and 48 hours. Conversely, no positive reactions were observed with the control group at any challenge concentration.

Table-1 Results of skin sensitization testing of Arbutin

Group	Challenge concentration	Hours after challenge exposure	Score								
			Erythema					Edema			
			0	1	2	3	4	0	1	2	3
Sensitization	10%	24	10	0	0	0	0	10	0	0	0
		48	10	0	0	0	0	10	0	0	0
	3%	24	10	0	0	0	0	10	0	0	0
		48	10	0	0	0	0	10	0	0	0
	1%	24	10	0	0	0	0	10	0	0	0
		48	10	0	0	0	0	10	0	0	0
Control	10%	24	10	0	0	0	0	10	0	0	0
		48	10	0	0	0	0	10	0	0	0
	3%	24	10	0	0	0	0	10	0	0	0
		48	10	0	0	0	0	10	0	0	0
	1%	24	10	0	0	0	0	10	0	0	0
		48	10	0	0	0	0	10	0	0	0

(Note) Sensitization group: Induction substance Arbutin, 10% solution
Challenge substance Arbutin, 10, 3 and 1% solutions in 50%, v/v, aqueous ethanol

Control group: Same procedures as the sensitization group using distilled water instead of the test substance solution. In the same manner as for the sensitization group, the test substance solutions were applied percutaneously at the challenge exposure.

Table-2 Results of skin sensitization testing with hydroquinone

Group	Challenge concentration	Hours after challenge exposure	Score								
			Erythema					Edema			
			0	1	2	3	4	0	1	2	3
Sensitization	10%	24	1	3	1	5	0	8	2	0	0
		48	0	1	2	6	1	8	1	0	1
	3%	24	2	3	2	3	0	9	1	0	0
		48	0	2	2	5	1	9	1	0	0
	1%	24	4	4	1	1	0	10	0	0	0
		48	1	3	4	2	0	10	0	0	0
Control	10%	24	10	0	0	0	0	10	0	0	0
		48	10	0	0	0	0	10	0	0	0
	3%	24	10	0	0	0	0	10	0	0	0
		48	10	0	0	0	0	10	0	0	0
	1%	24	10	0	0	0	0	10	0	0	0
		48	10	0	0	0	0	10	0	0	0

(Note) Sensitization group: Induction substance Hydroquinone, 5% solution
Challenge substance Hydroquinone, 10, 3 and 1% solutions in 50%, v/v, aqueous ethanol

Control group: Same procedures as the sensitization group using distilled water instead of the test substance solution. In the same manner as for the sensitization group, the test substance solutions were applied percutaneously at the challenge exposure.

Table-3 Result of skin sensitization testing with DNCB

Group	Challenge concentration	Hours after challenge exposure	Score								
			Erythema					Edema			
			0	1	2	3	4	0	1	2	3
Sensitization	0.1%	24	0	0	0	5	0	3	2	0	0
		48	0	0	0	5	0	4	1	0	0
	0.01%	24	2	3	0	0	0	5	0	0	0
		48	2	3	0	0	0	5	0	0	0
Control	0.1%	24	5	0	0	0	0	5	0	0	0
		48	5	0	0	0	0	5	0	0	0
	0.01%	24	5	0	0	0	0	5	0	0	0
		48	5	0	0	0	0	5	0	0	0

(Note) Sensitization group: Induction substance DNCB, 0.1% solution in liquid paraffin
Challenge substance DNCB, 0.1% and 0.01% solutions in acetone

Control group: Same procedures as the sensitization group using distilled water instead of the test substance solution. In the same manner as for the sensitization group, the test substance solutions were applied percutaneously at the challenge exposure.

4. Conclusion

Skin sensitization of Arbutin was evaluated in the guinea pig maximization test. No skin reactions were observed in animals in the sensitization group. Consequently, it is concluded that Arbutin does not possess sensitizing potential under the test conditions.

Hydroquinone used for the comparative substance induced positive reactions in the sensitization group.

References

1) Bentley-Phillips. B. and Bayles, M.A.H.,: Cutaneous reactions to topical application of hydroquinone. S. Afr. Med. J., 49: 1391-1395, 1975
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4) Magnusson, B. & Kilgman, A.M., Allergic Contact Dermatitis in the Guinea pig, Identifications of Contact Allergen: Springfield, Ill., C.C. Thomas, 1970

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TNO report

V4768

In vitro percutaneous absorption of [^{14}C]arbutin using human skin membranes

Date	11 February 2003
Authors	Dr.ir J.J.M. van de Sandt Ir. W.J.M. Maas
At request of	Shiseido Research Center Safety Research Laboratories 2-12-1, Fukuura, Kanazawa-ku Yokohama-shi 236-8643 Japan
TNO Project number	010.45162
TNO Study number	4768
Sponsor Study code	-
Status report	Final
Number of pages	37
Number of tables	3
Number of appendices	2

Summary

Arbutin was examined for *in vitro* percutaneous absorption through human skin membranes in three independent experiments. Arbutin was applied as an ingredient of two creams (CPB, BOP) and one gel, each at two concentrations: 3.0 % (low - L) and 6.3 % (high - H). Both freshly isolated (experiment 2) and cryopreserved skin tissue (experiments 1 and 3) was used.

After 24 h exposure, the mean relative amount of radioactivity reaching the receptor fluid was very low: 0.0154 % (CPB-H), 0.0153 % (CPB-L), 0.0203 % (BOP-H), 0.0361 % (BOP-L), 0.0195 % (Gel-H) and 0.0339 % (Gel-L). The mean lag time was 0.8 h (CPB-H), 1.2 h (CPB-L), 0.9 h (BOP-H), 1.3 h (BOP-L), 0.7 h (Gel-H) and 1.4 h (Gel-L). The mean total absorption, defined as the radioactivity present in the receptor fluid, the exposed epidermis (excluding tape strips), the exposed dermis and the skin surrounding the exposure site (excluding tape strips) was 0.160 ± 0.255 % (CPB-H), 0.126 ± 0.060 % (CPB-L), 0.143 ± 0.083 % (BOP-H), 0.214 ± 0.114 % (BOP-L), 0.135 ± 0.066 % (Gel-H) and 0.164 ± 0.016 % (Gel-L). No large differences were observed between the three formulation types tested.

With respect to the absorption of the reference compound (testosterone), no considerable differences were observed based on flux constants and Kp-value between freshly isolated and cryopreserved skin. Freshly isolated skin appeared to be slightly less permeable to testosterone: the relative amount of radioactivity reaching the receptor fluid during 24 h was 1.8900 % (fresh skin) and 3.4718 % and 3.9152 % (both cryopreserved skin). The absorption profiles, the relative absorption and the flux constants were comparable to earlier results obtained in our laboratory.

In conclusion, the mean total absorption of radioactivity from the three formulation types used in the present study was very low, ranging from 0.126 to 0.214 % of the applied dose over a 24-h exposure period.

Contents

Statement of GLP compliance

We, the undersigned, hereby declare that this report constitutes a true and complete representation of the procedures followed and of the results obtained in this study by TNO Nutrition and Food Research, and that the study was carried out under our supervision. The study was carried out in accordance with the OECD Principles of Good Laboratory Practice.

Dr.ir. J.J.M. van de Sandt
(Study director)

11 February 2003
Date

Dr. J.P. Groten
(Management)

11 FEBRUARY 2003
Date

Quality Assurance Statement

On:	*In vitro* percutaneous absorption of [^{14}C]arbutin using human skin membranes
Report Number:	V4768
Date :	11 February 2003

The protocol was audited as follows:

Date of audit:	Date of report:
8 August 2002	12 August 2002

The experimental phase of this study was audited by the Quality Assurance Unit of TNO Nutrition and Food Research as follows:

Date of audit:	Date of report:
8 August 2002	12 August 2002
3 September 2002	3 September 2002
4 September 2002	4 September 2002
6 September 2002	6 September 2002

This report was audited as follows:

Dates of audit:	Date of report:
6 January 2003 (draft report)	13 January 2003
12 February 2003	12 February 2003

I, the undersigned, hereby declare that this report provides an accurate record of the procedures employed and the results obtained in this study; all inspections were reported to the study director and the management on the dates indicated.

Drs. M.C.T.J. Meeuwsen
(Quality Assurance Auditor)

12 February 2003.
Date:

GLP compliance monitoring unit statement

inspectie W&V

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF
GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 88/320/EEC the conformity with the OECD Principles of GLP was assessed on 22-26 November1999 at

TNO Nutrition and Food Research Institute
Utrechtseweg 48
P.O. Box 360
3700 AJ Zeist

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Toxicity and Mutagenicity studies, and studies on Metabolism and Kinetics.

The Hague, 23 December 1999

Th. Helder, DVM
GLP Compliance Monitoring Unit

Inspectorate for Health Protection, Commodities and Veterinary Public Health
Ministry of Health, Welfare and Sport

Testing facility

The study was conducted by:

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Monitor:	Hitoshi Sasa

[1] Department of Biomolecular Sciences

1. Introduction

The objective of this study was to determine the *in vitro* percutaneous absorption of arbutin using human skin membranes. Six different formulations were compared. Testosterone was used as a reference compound with known *in vitro* absorption characteristics.

The protocol was drafted based on the SCCNFP guidelines for *in vitro* methods to assess percutaneous absorption of cosmetic ingredients (2000), the OECD guideline for the testing of chemicals (Draft Guideline 428: Skin absorption, in vitro method, December 2000), the ECETOC recommendations (1993), the report of ECVAM workshop 13 (1996) and the COLIPA test guidelines for *in vitro* assessment of dermal absorption and percutaneous penetration of cosmetic ingredients (Diembeck *et al.*, 1999).

The study was conducted according to the Organization for Economic Co-operation and Development. OECD Principles of Good Laboratory Practice (as revised in 1997), Paris, ENV/MC/CHEM (98)17.

2. Experimental

2.1 Test substance

2.1.1 General information

Name	: arbutin
Product category	: cosmetic ingredient (skin whitening agent)
Chemical name	: hydroquinone-β-D-glucopyranoside
CAS registry number	: 497-76-7
Emperical formula	: $C_{12}H_{16}O_7$
Molecular weight	: 272.25
Log Po/w	: -1.35
Solubility in water	: high

2.1.2 Radiolabeled arbutin

Shiseido Research Center has used the following [^{14}C]arbutin for the preparation of the test samples (see section 2.2):

Specific activity	: 3.44 MBq/mg
Radiochemical purity	: 97.7 % (TLC), 97.6 % (HPLC)
Batch number	: CP-2645

2.2 Test samples

The sponsor provided six different [^{14}C]-labelled formulations with suitable specific radioactivity. The formulations arrived 23 May 2002 and were stored at the testing facility at room temperature in the dark until use. The following formulations were provided:

Group A1

Name	: Cream-CPB-H
Batch number	: S-1601
Concentration arbutin	: 6.3 %
Specific activity arbutin	: 1.85 MBq/mg
Storage conditions	: room temperature
Expiration date	: 11 May 2005
Supplier	: Shiseido Research Center
TNO internal reference no. (Radioactive materials)	: 618

Group A2

Name	: Cream-CPB-L
Batch number	: S-1602
Concentration arbutin	: 3.0 %
Specific activity arbutin	: 3.50 MBq/mg
Storage conditions	: room temperature
Expiration date	: 11 May 2005
Supplier	: Shiseido Research Center
TNO internal reference no. (Radioactive materials)	: 619

Group B1

Name	: Cream-BOP-H
Batch number	: S-1603
Concentration arbutin	: 6.3 %
Specific activity arbutin	: 1.84 MBq/mg
Storage conditions	: room temperature
Expiration date	: 11 May 2005
Supplier	: Shiseido Research Center
TNO internal reference no. (Radioactive materials)	: 620

Group B2

Name	: Cream-BOP-L
Batch number	: S-1604
Concentration arbutin	: 3.0 %
Specific activity arbutin	: 3.50 MBq/mg
Storage conditions	: room temperature
Expiration date	: 11 May 2005
Supplier	: Shiseido Research Center
TNO internal reference no. (Radioactive materials)	: 621

Group C1

Name	: Gel-H
Batch number	: S-1605
Concentration arbutin	: 6.3 %
Specific activity arbutin	: 1.83 MBq/mg
Storage conditions	: room temperature
Expiration date	: 11 May 2005
Supplier	: Shiseido Research Center
TNO internal reference no. (Radioactive materials)	: 622

Group C2

Name	: Gel-L
Batch number	: S-1606
Concentration arbutin	: 3.0 %
Specific activity arbutin	: 3.50 MBq/mg
Storage conditions	: room temperature
Expiration date	: 11 May 2005
Supplier	: Shiseido Research Center
TNO internal reference no. (Radioactive materials)	: 623

All test samples were prepared by the sponsor. Before the start of each percutaneous absorption experiment, TNO has checked the radioactive concentration and homogeneity of the test samples. After taking samples from the vials, the vials were centrifuged in order to store the remaining test sample in the bottom of the vials.

2.3 Reference substances

Radiolabeled water	: $[^3H]H_2O$
Molecular weight	: 18.0
Specific Activity	: 37.0 MBq/g
Appearance	: clear liquid
Lot no.	: 3467348
Storage conditions	: 2-10 °C
Arrival date	: 1 July 2002
Expiration date	: 1 July 2003
Supplier	: PerkinElmer Life Sciences, Inc. (formerly: NEN™ Life Science Products)
TNO internal reference no. (Radioactive materials)	: 636

Name of the test substance	: Testosterone
Chemical name	: 4-androsten-17β-ol-3-one
Molecular weight	: 288.4
Batch no.	: H234
CAS. reg. no.	: 58-22-0
Storage conditions	: 2-10 °C
Arrival date	: 7 January 2000
Expiration date	: December 2004
Supplier	: Steraloids Inc. (Newport R.I., USA)
TNO internal reference no.	: 990365

Radiolabeled testosterone	: [4-^{14}C]testosterone
Specific Activity	: 1.983 GBq/mmol
Purity	: > 97 %
Lot no.	: 3379017
Appearance	: clear liquid (ethanol solution)
Storage conditions	: 2-10 °C
Supplier	: PerkinElmer Life Sciences, Inc. (formerly: NEN™ Life Science Products)
Arrival date	: 5 February 2002
Expiration date	: 5 February 2003
TNO internal reference no. (Radioactive materials)	: 598

For the reference group (group D), radiolabeled and non-radiolabeled testosterone were mixed in ethanol, yielding a concentration of 1.03 mg/mL (1.88 MBq/mL), 1.01 mg/mL (1.76 MBq/mL) and 1.05 mg/mL (2.04 MBq/mL), for experiments 1, 2 and 3 respectively. Of all test samples, the exact radioactivity was determined in mock doses prior to and after administration and was used for calculating the exact doses that were applied to the skin membranes.

2.4 Time schedule

The experimental phase of the study was performed as follows:
Experiment 1: 7 - 9 August 2002
Experiment 2: 14 - 16 August 2002
Experiment 3: 3 - 7 September 2002
Analysis of radioactivity in the samples was carried out until 10 September 2002.

2.5 Preparation of skin membranes

Human skin was obtained from three female Caucasian donors undergoing abdominal surgery at the University Medical Center (Utrecht, the Netherlands). Experiments with both fresh and frozen skin were performed.

For experiment 1, frozen human skin was used which was obtained from abdominal surgery of a 57 years old female donor (code TNA 07/02). Upon arrival at the laboratories of TNO Nutrition and Food Research on 23 July 2002, subcutaneous fat was removed and the skin was stored in aluminium foil at < -18 °C until use.

For experiment 2, fresh human skin was obtained directly after abdominal surgery of a 34 years old female donor (code TNA 09/02). The skin was transported to the laboratory on ice in a plastic container and the preparation of the fresh human skin membranes took place immediately after arrival at the laboratory.

For experiment 3, frozen human skin was used which was obtained from abdominal surgery of a 36 years old female donor (code TNA10/02). Upon arrival at the laboratories of TNO Nutrition and Food Research on 21 August 2002, subcutaneous fat was removed and the skin was stored in aluminium foil at < -18 °C until use.

Approval for the use of human skin for *in vitro* studies has been given by the TNO Medical Ethics Committee (MEC-TNO code 95/17).

Upon thawing of the skin, discs with a diameter of 16 mm were punched out. Part of the dermis was removed with scissors until a skin thickness of 0.7 - 0.9 mm was reached. The thickness of all skin membranes was measured with a digimatic micrometer (Mitutoyo Corporation, Japan). The mean skin thickness was 0.875 ± 0.028 mm (exp.1), 0.793 ± 0.056 (exp.2) and 0.848 ± 0.040 mm (exp.3).

2.6 Flow-through diffusion cells

The skin membranes were placed in 9 mm flow-through automated diffusion cells (PermeGear Inc., Riegelsville, PA, USA). The exposure area of the skin membranes in these cells was 0.64 cm^2. The temperature of the cells was

ca 32 °C, at ambient humidity. The receptor fluid was pumped at a speed of approximately 1.5 mL/h and consisted of a mixture of Dulbecco's Minimum Eagle Medium (DMEM) and Ham F12 culture medium (3:1) supplemented with Epidermal Growth Factor (EGF), (10 μg/L), hydrocortisone (400 μg/L), gentamicin (50 mg/L) and Fetal Calf Serum (10%, w/w). During the experiment, the receptor fluid was continuously gassed with 95% O_2 and 5% CO_2.

2.7 Experimental design

The study was conducted according to protocol P4768 entitled "*In vitro* percutaneous absorption of [^{14}C]arbutin using human skin membranes, approved by the study director on 5 August 2002 and by the sponsor on 6 August 2002.

Integrity of the membranes was assessed by determining the permeability coefficient (Kp) of tritiated water. Subsequently, arbutin was applied topically to the membranes as ingredient of 6 formulations. Testosterone was used as reference substance. The doses applied to the human membranes were as follows:

Group (test sample)	Experiment/ replicate	Amount formulation applied	Amount arbutin-/testosterone applied
A1 (cream-CPB-H)	1-1	1.7 mg/membrane	190.48 μg/cm^2
	1-2	1.7 mg/membrane	190.48 μg/cm^2
	2-1	2.8 mg/membrane	319.31 μg/cm^2
	2-2	2.1 mg/membrane	239.48 μg/cm^2
	3-1	1.4 mg/membrane	151.41 μg/cm^2
	3-2	2.5 mg/membrane	270.37 μg/cm^2
A2 (cream-CPB-L)	1-1	2.0 mg/membrane	99.33 μg/cm^2
	1-2	1.7 mg/membrane	84.43 μg/cm^2
	2-1	1.5 mg/membrane	77.16 μg/cm^2
	2-2	2.2 mg/membrane	113.17 μg/cm^2
	3-1	2.5 mg/membrane	124.13 μg/cm^2
	3-2	2.5 mg/membrane	124.13 μg/cm^2
B1 (cream-BOP-H)	1-1	2.2 mg/membrane	229.80 μg/cm^2
	1-2	1.8 mg/membrane	188.02 μg/cm^2
	2-1	2.0 mg/membrane	213.56 μg/cm^2
	2-2	1.5 mg/membrane	160.17 μg/cm^2
	3-1	3.5 mg/membrane	366.49 μg/cm^2
	3-2	2.8 mg/membrane	293.19 μg/cm^2

B2 (cream-BOP-L)	1-1	1.7 mg/membrane	82.90 µg/cm²
	1-2	1.9 mg/membrane	92.65 µg/cm²
	2-1	2.2 mg/membrane	110.38 µg/cm²
	2-2	1.8 mg/membrane	90.31 µg/cm²
	3-1	3.3 mg/membrane	166.43 µg/cm²
	3-2	1.7 mg/membrane	85.73 µg/cm²
C1 (gel-H)	1-1	2.0 mg/membrane	209.95 µg/cm²
	1-2	1.4 mg/membrane	146.97 µg/cm²
	2-1	2.4 mg/membrane	251.33 µg/cm²
	2-2	2.4 mg/membrane	251.33 µg/cm²
	3-1	2.5 mg/membrane	267.43 µg/cm²
	3-2	2.2 mg/membrane	235.34 µg/cm²
C2 (gel-L)	1-1	2.3 mg/membrane	113.83 µg/cm²
	1-2	2.0 mg/membrane	98.99 µg/cm²
	2-1	2.1 mg/membrane	101.84 µg/cm²
	2-2	2.0 mg/membrane	96.99 µg/cm²
	3-1	1.8 mg/membrane	88.01 µg/cm²
	3-2	3.0 mg/membrane	146.68 µg/cm²
D (testosterone in ethanol*)	1-1	10 µL/membrane	16.35 µg/cm²
	1-2	10 µL/membrane	16.35 µg/cm²
	2-1	10 µL/membrane	15.72 µg/cm²
	2-2	10 µL/membrane	15.72 µg/cm²
	3-1	10 µL/membrane	15.98 µg/cm²
	3-2	10 µL/membrane	15.98 µg/cm²

* ethanol was carefully evaporated using pressurized air

2.8 Integrity of skin membranes

The inner side of donor compartment was dried with a sterile gauze swab and 200 µl saline containing tritiated water (experiment 1: 15.9 kBq/mL, experiment 2: 19.9 kBq/mL, experiment 3: 16.1 kBq/mL) was applied in the donor compartment of the flow-through diffusion cells. The donor compartment was covered with a glass slide. Samples of the receptor fluid were collected every hour up to three hours after application. Subsequently, the tritiated water remaining at the application site was removed with a sterile gauze swab. Only skin membranes with a permeability coefficient (Kp) of less than 1.98×10^{-3} cm.h^{-1} for tritiated water were used for the assessment of percutaneous absorption of arbutin and testosterone. When membranes did not meet this criteria, they were replaced by new membranes. If one or two membranes again did not meet the criteria for membrane integrity, group D was omitted from the experiment.

2.9 Percutaneous absorption of arbutin

Arbutin and testosterone were applied to the skin membranes approximately 12 h after removal of tritiated water. The formulations and the ethanol solution were applied using a disposable spatula and a pipet, respectively. In all groups, the receptor fluid was collected at the following intervals for determination of total radioactivity: 0-1 h, 1-2 h , 2-4, 4-6 and 6-8 h, followed by 4-h intervals until 24 h.

2.10 Determination of tissue distribution

The tissue distribution of the radioactivity was determined 24 h after application of the test samples. To this purpose, the receptor fluid was collected and the receptor compartment of the diffusion cell was washed two times with 1.0 mL water. The remaining test compound was removed from the application and non-application sites, each with 5 cotton swabs soaked in 3 % aqueous Teepol solution, 1 cotton swab soaked in water and 1 filter paper. Subsequently, the exposed area of each skin membrane was tape-stripped using D-squame (Monoderm, Monaco) (maximally 20 times per membrane; every 2 tape strips were pooled). The remaining exposed epidermis was then be isolated by heat separation (*ca* 15 seconds on a heating block of *ca* 60 °C). Then, each skin membrane was separated in the exposed and non-exposed area using a 8-mm punch biopsy needle. The non-exposed skin area was tape stripped using cellophane tape (Nichiban, Japan) (5 times per membrane). Total radioactivity was determined in all compartments separately.

2.11 Determination of radioactivity

Radioactivity was determined in samples of dose solutions, receptor fluid, cotton swabs, tape strips, epidermal and dermal fractions. Exact procedures are described in facility SOP's. The SOP numbers used were retained in the study files.

Test samples	Aliquots of the radiolabeled test samples were diluted in water. Aliquots of the resulting solution were added directly to liquid scintillant (Ultima Gold™) and measured by liquid scintillation counting.
Receptor fluid	Samples of the receptor fluid were added directly to a liquid scintillant (Ultima Gold™).
Cotton swabs/filter paper	Cotton swabs and filter paper were added directly to a liquid scintillant (Ultima Gold™).
Skin tissue	Aliquots of the digested epidermis and dermis were added to an appropriate liquid scintillant (Hionic Fluor-™) and measured by liquid scintillation counting.
Tape strips	Tape strips (2 per vial) were added directly to a liquid scintillant (Ultima Gold™).

Radioactivity	Radioactivity in all samples was determined by liquid scintillation counting (LSC) using DOT-DPM (Digital Overlay Technique using the Spectrum Library and the External Standard Spectrum) for quench correction on a Wallac Pharmacia S1409 scintillation counter.
Background samples	Background values were measured with each sample sequence using the respective scintillation mixture without any samples.

The recovery of the total radioactivity of each replicate membrane must be 100 ± 15 %. Limits of determination (LQ) for radioactivity in receptor fluid, cotton swabs, skin extracts and skin pellets were calculated using the value of double background as limit of quantification. Calibration procedures for the instruments were established at the testing facilities.

2.12 Calculations

- The cumulative penetration of test substance equivalents was calculated from the receptor fluid samples by the following equation:

  Cumulative $DPM_T = DPM_T + \Sigma(DPM_{T-1} \ldots DPM_1)$

  DPM_T : radioactivity at sampling time T

  DPM_{T-1} : radioactivity at the sampling time preceding T

  DPM_1 : radioactivity at the first sampling time
- The cumulative absorption, expressed as percentage of the dose applied
- The flux constant [$\mu g \times cm^{-2} \times h^{-1}$] was calculated from the linear portion of the cumulative penetration curve as follows:

  Flux constant = $\Delta C_{Tx-Ty} / (x-y)$

  ΔC_{Tx-Ty} : increase in penetrant concentration during the linear portion of the curve

  x : begin of linear portion of the curve

  y : end of linear portion of the curve
- The permeability coefficient or Kp value [$cm \times h^{-1}$] was calculated as follows:

  Kp = flux constant [$\mu g \times cm^{-2} \times h^{-1}$]/applied concentration [$\mu g \times cm^{-3}$]
- Lag time [h] was obtained by extrapolating the linear portion of the cumulative penetration curves to the x-axis
- Mass balance
- Total relative absorption [% of dose applied]: radioactivity in the receptor fluid (receptor fluid samples and receptor compartment), the exposed epidermis (excluding tape strips), the exposed dermis and the skin surrounding the exposure site (excluding tape strips)

All data have been reported in the present report (see appendices), but data points which deviate more than 3 times from the mean value were excluded from the calculations.

2.13 Retention of records

The raw data, the master copy of the final report and all other information relevant to the quality and integrity of the study were retained in the archives of the TNO Nutrition and Food Research for a period of at least 15 years after reporting of the study.

2.14 Deviations from the protocol

- One skin membrane (group B2, replicate 1 of experiment 1) was used in the study with a Kp value slightly higher than the cut-off described in the protocol: 1.98×10^{-3} instead of 1.95×10^{-3} cm/h.
- In experiments 1 and 3, part of the the epidermis was removed during tape stripping of some skin membranes. Therefore, less than 20 tape strips were used for these membranes.
- The total relative absorption of arbutin was calculated.

This deviation is considered not to have negatively influenced the validity and outcome of the study.

3. Results

3.1 Integrity of skin membranes

Prior to the determination of the percutaneous absorption of arbutin and the reference compound (testosterone), the permeability coefficient (Kp) for tritiated water was determined in the human skin membranes.

Membranes with a Kp value below the cut-off values of 1.95×10^{-3} cm.h^{-1} were selected for the study with the exception of the skin membrane used for group B2, replicate 1 of experiment 1, which had a slightly higher Kp (1.98×10^{-3} cm.h^{-1}). The individual data of the penetration of tritiated water through the selected skin membranes are given in Appendix 1.

3.2 Percutaneous absorption of arbutin

Arbutin was examined for *in vitro* percutaneous absorption through human skin membranes in three independent experiments. Arbutin was applied as an ingredient of three formulation types, each at two concentrations: 3.0 % (low - L) and 6.3 % (high - H).

Experiment 1

In one of the membranes (B1-1), an exceptionally high absorption of radioactivity was observed, in comparison to the other membranes. Therefore, this membrane was excluded from the calculations. Furthermore, the data from membrane C2-1 were excluded from the calculations since the total (relative) absorption was higher than 3 times the mean value for three experiments (see section 2.12). This was mainly caused by exceptionally high levels remaining in the dermis and rest skin.

After 24 h exposure, the relative amount of radioactivity reaching the receptor fluid was 0.0006 % (CPB-H), 0.0041 % (CPB-L), 0.0020 % (BOP-H, only membrane B1-2), 0.0034 % (BOP-L), 0.0113 % (Gel-H) and 0.0042 % (Gel-L, only membrane C2-2). The flux constants were 0.0005 μg.cm^{-2}.h^{-1} (CPB-H), 0.0005 μg.cm^{-2}.h^{-1} (CPB-L), 0.0005 μg.cm^{-2}.h^{-1} (BOP-H, only membrane B1-2), 0.0005 μg.cm^{-2}.h^{-1} (BOP-L), 0.0014 μg.cm^{-2}.h^{-1} (Gel-H) and 0.0006 μg.cm^{-2}.h^{-1} (Gel-L, only membrane C2-2). The lag time was 1.0 h (CPB-H), 0.9 h (CPB-L), 1.0 h (BOP-H, only membrane B1-2), 0.7 h (BOP-L), 0.8 h (Gel-H) and 3.5 h (Gel-L, only membrane C2-2) (appendices 2 and 3).

At the end of the exposure period, the tissue distribution of the radioactivity was determined. For all groups, clearly most of the radioactivity could be removed from the application site using cotton swabs. The total absorption, defined as the radioactivity present in the receptor fluid, the exposed epidermis (excluding tape strips), the exposed dermis and the skin surrounding the exposure site (excluding tape strips), was 0.015 % (CPB-H), 0.094 % (CPB-L), 0.032 % (BOP-H, only

membrane B1-2), 0.095 % (BOP-L), 0.213 % (Gel-H) and 0.147 % (Gel-L, only membrane C2-2).

In some skin membranes, part of the epidermis of the skin membranes was removed during tape stripping (A1-2: tape strip no. 14, B2-2: tape strip no. 9, C2-2: tape strip no. 17), and was added to the vial containing the remaining epidermis. Therefore, a (small) part of the amount of the test substance recovered from the epidermis may be considered located in the stratum corneum and thus as non-absorbed.

The total recovery of the radioactivity in this experiment ranged between 93.3 % and 111.5 %.

Experiment 2

In one of the membranes (A1-1), an exceptionally high absorption of radioactivity was observed, in comparison to the other membranes. Therefore, this membrane was excluded from the calculations.

After 24 h exposure, the relative amount of radioactivity reaching the receptor fluid was 0.0031 % (CPB-H, only membrane A1-2), 0.0120 % (CPB-L), 0.0124 % (BOP-H), 0.0268 % (BOP-L), 0.0184 % (Gel-H) and 0.0411 % (Gel-L). The flux constants were 0.0007 $\mu g.cm^{-2}.h^{-1}$ (CPB-H), 0.0005 $\mu g.cm^{-2}.h^{-1}$ (CPB-L), 0.0011 $\mu g.cm^{-2}.h^{-1}$ (BOP-H), 0.0012 $\mu g.cm^{-2}.h^{-1}$ (BOP-L), 0.0024 $\mu g.cm^{-2}.h^{-1}$ (Gel-H) and 0.0018 $\mu g.cm^{-2}.h^{-1}$ (Gel-L). The lag time was 0.9 h (CPB-H, only membrane A1-2), 0.8 h (CPB-L), 1.0 h (BOP-H), 2.6 h (BOP-L), 1.3 h (Gel-H) and 1.7 h (Gel-L) (appendices 2 and 3).

At the end of the exposure period, the tissue distribution of the radioactivity was determined. For all groups, clearly most of the radioactivity could be removed from the application site using cotton swabs. The total absorption, defined as the radioactivity present in the receptor fluid, the exposed epidermis (excluding tape strips), the exposed dermis and the skin surrounding the exposure site (excluding tape strips), was 0.613 % (CPB-H, only membrane A1-2), 0.155 % (CPB-L), 0.202 % (BOP-H), 0.223 % (BOP-L), 0.079 % (Gel-H) and 0.175 % (Gel-L).

The total recovery of the radioactivity in this experiment ranged between 86.8 % and 106.4 %.

Experiment 3

After 24 h exposure, the relative amount of radioactivity reaching the receptor fluid was 0.0364 % (CPB-H), 0.0298 % (CPB-L), 0.0372 % (BOP-H), 0.0782 % (BOP-L), 0.0289 % (Gel-H) and 0.0415 % (Gel-L). The flux constants were 0.0032 $\mu g.cm^{-2}.h^{-1}$ (CPB-H), 0.0017 $\mu g.cm^{-2}.h^{-1}$ (CPB-L), 0.0050 $\mu g.cm^{-2}.h^{-1}$ (BOP-H), 0.0039 $\mu g.cm^{-2}.h^{-1}$ (BOP-L), 0.0029 $\mu g.cm^{-2}.h^{-1}$ (Gel-H) and 0.0020 $\mu g.cm^{-2}.h^{-1}$ (Gel-L) (appendices 2 and 3). The lag time was 0.5 h (CPB-H), 1.9 h (CPB-L), 0.8 h (BOP-H), 0.5 h (BOP-L), 0.0 h (Gel-H) and 0.0 h (Gel-L).

At the end of the exposure period, the tissue distribution of the radioactivity was determined. For all groups, clearly most of the radioactivity could be removed from

the application site using cotton swabs. The total absorption, defined as the radioactivity present in the receptor fluid, the exposed epidermis (excluding tape strips), the exposed dermis and the skin surrounding the exposure site (excluding tape strips), was 0.079 % (CPB-H), 0.130 % (CPB-L), 0.139 % (BOP-H), 0.323 % (BOP-L), 0.112 % (Gel-H) and 0.163 % (Gel-L).

In almost all skin membranes, part of the epidermis of the skin membranes was removed during tape stripping (A1-2: tape strip no. 14, A2-1: tape strip no. 6, A2-2: tape strip no. 9, B1-1: tape strip no. 13, B1-2: tape strip no. 17, B2-2: tape strip no. 15, C1-2: tape strip no. 10, C2-1: tape strip no. 11, C2-2: tape strip no. 16), and was added to the vial containing the remaining epidermis. Therefore, at least a (small) part of the amount of the test substance recovered from the epidermis may be considered located in the stratum corneum and thus as non-absorbed.

The total recovery of the radioactivity in this experiment ranged between 91.1 % and 103.2 %.

An overview of the mean data from the three experiments is presented in Table 1.

Table 1 Overview table of the *in vitro* percutaneous absorption of arbutin through human skin membranes

Group	A1 CPB-H		A2 CPB-L		B1 BOP-H	
Dose [$\mu g.cm^{-2}$]	208.4		103.7		244.3	
n	5		6		5	
Penetration within [h]	% of dose	$\mu g.cm^{-2}$	% of dose	$\mu g.cm^{-2}$	% of dose	$\mu g.cm^{-2}$
24	0.0154	0.0321	0.0153	0.0174	0.0203	0.0584
Flux constant [$\mu g.cm^{-2}.h$]	0.0016		0.0009		0.0025	
Kp value [$cm.h^{-1}$] x 10^{-6}	0.0256		0.0295		0.0403	
Lag time [h]	0.8		1.2		0.9	
Total absorption* [% of dose]	0.160		0.126		0.143	

Group	B2 BOP-L		C1 Gel-H		C2 Gel-L	
Dose [$\mu g.cm^{-2}$]	104.7		227.1		106.5	
n	6		6		5	
Penetration within [h]	% of dose	$\mu g.cm^{-2}$	% of dose	$\mu g.cm^{-2}$	% of dose	$\mu g.cm^{-2}$
24	0.0361	0.0406	0.0195	0.0477	0.0339	0.0384
Flux constant [$\mu g.cm^{-2}.h$]	0.0019		0.0022		0.0017	
Kp value [$cm.h^{-1}$] x 10^{-6}	0.0625		0.0355		0.0553	
Lag time [h]	1.3		0.7		1.4	
Total absorption* [% of dose]	0.214		0.135		0.164	

* radioactivity present in receptor fluid, exposed epidermis (excluding tape strips), exposed dermis and skin surrounding the exposure site (excluding tape strips).

3.3 Percutaneous absorption of the reference compound

Testosterone was used as a reference compound in experiment 1, 2 and 3, and was applied at a dose of 16.35 $\mu g.cm^{-2}$ (experiment 1), 15.72 $\mu g.cm^{-2}$ (experiment 2) and 15.98 $\mu g.cm^{-2}$ (experiment 3). The exposure time was 24 hours.

Experiment 1

The mean amount that reached the receptor fluid after 24 h was 0.5677 $\mu g.cm^{-2}$. The lag time was 7.5 h and the mean recovery of the radioactivity was 96.6 % (Table 2, Appendices 2 and 4).

Experiment 2

The mean amount that reached the receptor fluid after 24 h was 0.2970 $\mu g.cm^{-2}$. The lag time was 8.7 h and the mean recovery of the radioactivity was 100.4 % (Table 2, Appendices 2 and 4).

Experiment 3

Membrane D2 was excluded from the calculations due to the low recovery value (73.8 % of the dose). The amount that reached the receptor fluid after 24 h was 0.6257 $\mu g.cm^{-2}$. The lag time was 11.3 h and the recovery of the radioactivity was 94.7 % (Table 2, Appendices 2 and 4).

Table 2 Overview table of the *in vitro* percutaneous absorption of testosterone through human membranes

Experiment	1		2		3	
Dose [$\mu g.cm^{-2}$]	16.35		15.72		15.98	
n	2		2		1	
Absorption within [h]	% of dose	$\mu g.cm^{-2}$	% of dose	$\mu g.cm^{-2}$	% of dose	$\mu g.cm^{-2}$
24	3.4718	0.5677	1.8900	0.2970	3.9152	0.6257
Flux constant [$\mu g.cm^{-2}.h^{-1}$]	0.0328		0.0179		0.0487	
Kp value [$cm.h^{-1}$] x 10^{-3}	0.031		0.018		0.048	
Lag time [h]	7.5		8.7		11.3	

4. Discussion and conclusion

Arbutin was examined for *in vitro* percutaneous absorption through human skin membranes in three independent experiments. Arbutin was applied as an ingredient of three formulation types, each at two concentrations: 3.0 % (low - L) and 6.3 % (high - H). Both freshly isolated (experiment 2) and cryopreserved skin tissue (experiments 1 and 3) was used.

After 24 h exposure, the mean relative amount of radioactivity reaching the receptor fluid was very low: 0.0154 % (CPB-H), 0.0153 % (CPB-L), 0.0203 % (BOP-H), 0.0361 % (BOP-L), 0.0195 % (Gel-H) and 0.0339 % (Gel-L). The mean flux constants were 0.0016 $\mu g.cm^{-2}.h^{-1}$ (CPB-H), 0.0009 $\mu g.cm^{-2}.h^{-1}$ (CPB-L), 0.0025 $\mu g.cm^{-2}.h^{-1}$ (BOP-H), 0.0019 $\mu g.cm^{-2}.h^{-1}$ (BOP-L), 0.0022 $\mu g.cm^{-2}.h^{-1}$ (Gel-H) and 0.0017 $\mu g.cm^{-2}.h^{-1}$ (Gel-L). The mean lag time was 0.8 h (CPB-H), 1.2 h (CPB-L), 0.9 h (BOP-H), 1.3 h (BOP-L), 0.7 h (Gel-H) and 1.4 h (Gel-L).

The mean total absorption, defined as the radioactivity present in the receptor fluid, the exposed epidermis (excluding tape strips), the exposed dermis and the skin surrounding the exposure site (excluding tape strips) was 0.160 ± 0.255 % (CPB-H), 0.126 ± 0.060 % (CPB-L), 0.143 ± 0.083 % (BOP-H), 0.214 ± 0.114 % (BOP-L), 0.135 ± 0.066 % (Gel-H) and 0.164 ± 0.016 % (Gel-L). No large differences were observed between the three formulation types tested.

The data from membranes B1-1 (experiment 1) and A1-1 (experiment 2) were excluded from the calculations since their absorption profiles clearly differed from its replicate in the same experiment and also from the profiles obtained in two additional experiments. Furthermore, the data from membrane C2-1 (experiment 1) were excluded since the total absorption clearly out-ranged the exclusion criteria (3 times the mean value). The high total absorption was mainly caused by exceptionally high levels remaining in the dermis and rest skin compared to the other membranes. The fact that the amount of test compound removed from the non-exposed area was relatively high (*ca* 7.6 % of the applied dose) indicates that exposure may have taken place over a larger skin surface than 0.64 cm^2. This may have resulted in higher levels of test compound in the dermis and rest skin.

With respect to the absorption of the reference compound (testosterone), no considerable differences were observed based on flux constants and Kp-value between freshly isolated and cryopreserved skin. Freshly isolated skin appeared to be slightly less permeable to testosterone. The absorption profiles, the relative absorption and the flux constants were comparable to earlier results obtained in our laboratory.

In conclusion, the mean total absorption of radioactivity from the three formulation types used in the present study was very low, ranging from 0.126 to 0.214 % of the applied dose over a 24-h exposure period.

5. References

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Appendices

Appendix 1 - Individual data of the membrane integrity test

Appendix 2 - Individual data of the cumulative absorption of arbutin and testosterone

Appendix 3 - Figures of the cumulative percutaneous absorption of arbutin

Appendix 4 - Individual data of the tissue distribution of arbutin and testosterone

Appendix 1 Individual data of the membrane integrity test

Table I **Cumulative penetration of tritiated water through human skin prior to application of the test samples (Experiment 1)**

Cumulative radioactivity [dpm]							
Cell number	1	2	3	4	5	6	7
Time interval							
0-1 h	256	223	230	239	328	118	471
0-2 h	1669	1167	1358	1249	1638	568	1858
0-3 h	3045	2332	2515	2425	3095	1128	3613
Penetration rate [dpm.cm^{-2}.h^{-1}]	1586	1215	1310	1263	1612	588	1882
Kp value [cm.h^{-1}]× 10^{-3}	1.67	1.28	1.38	1.33	1.69	0.62	1.98

Cumulative radioactivity [dpm]							
Cell number	8	9	10	11	12	13	14
Time interval							
0-1 h	71	446	265	250	120	299	299
0-2 h	470	1797	925	1157	813	1362	1339
0-3 h	991	3371	1671	2235	1534	2407	2562
Penetration rate [dpm.cm^{-2}.h^{-1}]	516	1756	870	1164	799	1254	1334
Kp value [cm.h^{-1}]× 10^{-3}	0.54	1.84	0.91	1.22	0.84	1.32	1.40

Appendix 1 continued

Table II **Cumulative penetration of tritiated water through human skin prior to application of the test samples (Experiment 2)**

Cumulative radioactivity [dpm]							
Cell number	1	2	3	4	5	6	7
Time interval							
0-1 h	81	129	126	147	109	264	174
0-2 h	589	829	812	878	711	1340	952
0-3 h	1297	1801	1723	1721	1587	2517	1927
Penetration rate [$dpm.cm^{-2}.h^{-1}$]	676	938	897	896	827	1311	1004
Kp value [$cm.h^{-1}$]$\times 10^{-3}$	0.57	0.79	0.75	0.75	0.69	1.10	0.84

Cumulative radioactivity [dpm]							
Cell number	8	9	10	11	12	13	14
Time interval							
0-1 h	128	90	181	262	188	120	200
0-2 h	827	711	1090	905	1057	730	953
0-3 h	1756	1610	2249	1809	2086	1751	1949
Penetration rate [$dpm.cm^{-2}.h^{-1}$]	915	839	1171	942	1086	912	1015
Kp value [$cm.h^{-1}$]$\times 10^{-3}$	0.77	0.70	0.98	0.79	0.91	0.77	0.85

Appendix 1 continued

Table III Cumulative penetration of tritiated water through human skin prior to application of the test samples (Experiment 3)

Cumulative radioactivity [dpm]							
Cell number	1	2	3	4	5	6	7
Time interval							
0-1 h	96	220	210	245	187	144	268
0-2 h	710	988	870	1132	864	756	1150
0-3 h	1412	2023	1789	2083	1759	1619	2233
Penetration rate [dpm.cm^{-2}.h^{-1}]	735	1054	932	1085	916	843	1163
Kp value [cm.h^{-1}]× 10^{-3}	0.76	1.09	0.96	1.12	0.95	0.87	1.20

Cumulative radioactivity [dpm]							
Cell number	8	9	10	11	12	13	14
Time interval							
0-1 h	129	172	150	114	145	167	86
0-2 h	813	928	939	776	903	803	649
0-3 h	1513	1687	1832	1653	1779	1599	1334
Penetration rate [dpm.cm^{-2}.h^{-1}]	788	879	954	861	927	833	695
Kp value [cm.h^{-1}]× 10^{-3}	0.81	0.91	0.98	0.89	0.96	0.86	0.72

Appendix 2 Individual data of the cumulative absorption of arbutin and testosterone

Table IV Individual data of the cumulative absorption of arbutin and testosterone (Experiment 1)

	Cumulative absorption (µg/cm2)													
Time (h)	A1-1	A1-2	A2-1	A2-2	B1-1	B1-2	B2-1	B2-2	C1-1	C1-2	C2-1	C2-2	D1	D2
1	0.0000	0.0000	0.0002	0.0000	0.0004	0.0000	0.0002	0.0000	0.0004	0.0000	0.0002	0.0002	0.0000	0.0000
2	0.0006	0.0004	0.0007	0.0003	0.0025	0.0005	0.0011	0.0002	0.0029	0.0009	0.0002	0.0002	0.0029	0.0022
4	0.0006	0.0004	0.0021	0.0003	0.0113	0.0005	0.0031	0.0002	0.0071	0.0009	0.0019	0.0002	0.0089	0.0066
6	0.0006	0.0004	0.0033	0.0003	0.0282	0.0005	0.0044	0.0002	0.0112	0.0009	0.0039	0.0019	0.0235	0.0156
8	0.0020	0.0004	0.0044	0.0003	0.0481	0.0005	0.0054	0.0002	0.0141	0.0009	0.0047	0.0027	0.0515	0.0328
12	0.0020	0.0004	0.0062	0.0003	0.0918	0.0005	0.0054	0.0002	0.0204	0.0009	0.0047	0.0027	0.1555	0.1100
16	0.0020	0.0004	0.0062	0.0003	0.1508	0.0005	0.0054	0.0002	0.0264	0.0009	0.0047	0.0027	0.2962	0.2268
20	0.0020	0.0004	0.0062	0.0003	0.1926	0.0005	0.0054	0.0002	0.0341	0.0009	0.0047	0.0027	0.4209	0.3668
24	0.0020	0.0004	0.0077	0.0003	0.2855	0.0038	0.0054	0.0002	0.0463	0.0009	0.0061	0.0041	0.6026	0.5326
Relative absorption (% at 24h)	0.0010	0.0002	0.0077	0.0004	0.1242	0.0020	0.0065	0.0003	0.0221	0.0006	0.0053	0.0042	3.6862	3.2584
Linear range	1-2	1-2	1-8	1-2	4-24	1-2	1-8	1-2	1-24	1-2	2-6	4-8	8-24	8-24
Flux constant (µg/cm²/h)	0.0006	0.0004	0.0006	0.0003	0.0132	0.0005	0.0008	0.0002	0.0019	0.0009	0.0009	0.0006	0.0342	0.0314
Kp * 10^{-6} (cm/h)	0.0090	0.0061	0.0206	0.0112	0.2098	0.0077	0.0251	0.0078	0.0294	0.0150	0.0307	0.0213	32.6684	30.0228
Lag time (h)	1.0	1.0	0.7	1.0	4.1	1.0	0.5	1.0	0.6	1.0	1.9	3.5	7.1	7.9
R^2	1.0000	1.0000	0.9985	1.0000	0.9800	1.0000	0.9804	1.0000	0.9897	1.0000	0.9981	0.9537	0.9912	0.9826
Mean rel. absorption (% at 24h)	0.0006		0.0041		0.0020		0.0034		0.0113		0.0042		3.4718	
Mean abs. absorption (µg/cm²/24h)	0.0012		0.0040		0.0038		0.0028		0.0236		0.0041		0.5677	
Mean flux constant (µg/cm²/h)	0.0005		0.0005		0.0005		0.0005		0.0014		0.0006		0.0328	
Mean Kp * 10^{-6} (cm/h)	0.0076		0.0159		0.0077		0.0165		0.0222		0.0213		31.3456	
Mean lag time (h)	1.0		0.9		1.0		0.7		0.8		3.5		7.5	

B1-1: Data not included in calculations due to clearly deviating absorption profile.
C2-1: Data not included in calculations due to clearly deviating levels of test compound in the skin. Total absorption > 3x mean value.

Table V Individual data of the cumulative absorption of arbutin and testosterone (Experiment 2)

	Cumulative absorption (µg/cm2)													
Time (h)	A1-1	A1-2	A2-1	A2-2	B1-1	B1-2	B2-1	B2-2	C1-1	C1-2	C2-1	C2-2	D1	D2
1	0.0002	0.0002	0.0001	0.0002	0.0000	0.0003	0.0001	0.0000	0.0000	0.0004	0.0002	0.0001	0.0002	0.0000
2	0.0009	0.0007	0.0004	0.0007	0.0006	0.0012	0.0007	0.0003	0.0007	0.0019	0.0010	0.0009	0.0005	0.0002
4	0.0047	0.0020	0.0011	0.0017	0.0026	0.0033	0.0027	0.0018	0.0039	0.0072	0.0036	0.0040	0.0005	0.0002
6	0.0127	0.0035	0.0019	0.0030	0.0056	0.0057	0.0047	0.0040	0.0089	0.0132	0.0073	0.0082	0.0023	0.0040
8	0.0250	0.0049	0.0030	0.0043	0.0083	0.0079	0.0069	0.0062	0.0141	0.0194	0.0106	0.0123	0.0072	0.0151
12	0.0581	0.0077	0.0050	0.0064	0.0132	0.0115	0.0106	0.0116	0.0228	0.0290	0.0180	0.0201	0.0293	0.0685
16	0.0994	0.0077	0.0067	0.0080	0.0174	0.0146	0.0145	0.0176	0.0315	0.0361	0.0258	0.0280	0.0743	0.1439
20	0.1439	0.0077	0.0081	0.0103	0.0221	0.0176	0.0178	0.0239	0.0401	0.0404	0.0351	0.0324	0.1394	0.2535
24	0.1865	0.0077	0.0098	0.0127	0.0260	0.0202	0.0211	0.0311	0.0486	0.0437	0.0444	0.0375	0.2073	0.3868
Rel. absorption (% at 24h)	0.0584	0.0031	0.0127	0.0112	0.0122	0.0126	0.0192	0.0345	0.0193	0.0174	0.0436	0.0386	1.3188	2.4612
Linear range	8-24	1-12	1-24	1-24	1-24	1-12	1-24	8-24	8-24	1-12	8-24	8-24	8-24	8-24
Flux constant (µg/cm²/h)	0.0102	0.0007	0.0004	0.0005	0.0012	0.0010	0.0009	0.0016	0.0022	0.0027	0.0021	0.0016	0.0126	0.0232
Kp * 10^{-6} (cm/h)	0.1622	0.0109	0.0145	0.0179	0.0185	0.0165	0.0311	0.0519	0.0343	0.0426	0.0706	0.0523	12.5352	23.0786
Lag time (h)	6.0	0.9	1.1	0.5	1.2	0.7	0.9	4.4	1.4	1.1	3.4	0.0	8.8	8.5
R^2	0.9973	0.9989	0.9973	0.9981	0.9980	0.9974	0.9987	0.9969	0.9999	0.9968	0.9972	0.9949	0.9623	0.9697
Mean rel. absorption (% at 24h)	0.0031		0.0120		0.0124		0.0268		0.0184		0.0411		1.8900	
Mean abs. absorption (µg/cm²/24h)	0.0077		0.0113		0.0231		0.0261		0.0461		0.0409		0.2970	
Mean flux constant (µg/cm²/h)	0.0007		0.0005		0.0011		0.0012		0.0024		0.0018		0.0179	
Mean Kp * 10^{-6} (cm/h)	0.0109		0.0162		0.0175		0.0415		0.0384		0.0614		17.6069	
Mean lag time (h)	0.9		0.8		1.0		2.6		1.3		1.7		8.7	

A1-1: Data not included in calculations due to clearly deviating absorption profile.

Appendix 2 continued

Table VI Individual data of the cumulative absorption of arbutin and testosterone (Experiment 3)

	Cumulative absorption (µg/cm2)													
Time (h)	A1-1	A1-2	A2-1	A2-2	B1-1	B1-2	B2-1	B2-2	C1-1	C1-2	C2-1	C2-2	D1	D2
1	0.0003	0.0007	0.0003	0.0003	0.0009	0.0004	0.0006	0.0003	0.0007	0.0004	0.0000	0.0005	0.0000	0.0000
2	0.0016	0.0041	0.0015	0.0015	0.0055	0.0054	0.0050	0.0029	0.0054	0.0039	0.0016	0.0057	0.0000	0.0000
4	0.0060	0.0129	0.0038	0.0042	0.0171	0.0199	0.0167	0.0095	0.0148	0.0108	0.0047	0.0164	0.0020	0.0000
6	0.0130	0.0221	0.0066	0.0076	0.0280	0.0352	0.0276	0.0179	0.0241	0.0176	0.0078	0.0257	0.0105	0.0045
8	0.0178	0.0297	0.0085	0.0110	0.0352	0.0455	0.0362	0.0242	0.0308	0.0224	0.0097	0.0324	0.0254	0.0137
12	0.0290	0.0462	0.0127	0.0194	0.0522	0.0707	0.0537	0.0361	0.0463	0.0316	0.0132	0.0461	0.1058	0.0622
16	0.0393	0.0616	0.0163	0.0276	0.0731	0.0905	0.0704	0.0502	0.0602	0.0409	0.0170	0.0579	0.2363	0.1409
20	0.0498	0.0763	0.0203	0.0363	0.0930	0.1063	0.0883	0.0638	0.0736	0.0490	0.0202	0.0696	0.4129	0.2515
24	0.0591	0.0913	0.0242	0.0497	0.1179	0.1240	0.1064	0.0793	0.0887	0.0578	0.0233	0.0829	0.6257	0.3793
Rel. absorption (% at 24h)	0.0390	0.0338	0.0195	0.0401	0.0322	0.0423	0.0639	0.0925	0.0332	0.0246	0.0264	0.0565	3.9152	2.3734
Linear range	8 - 24	8 - 24	8 - 24	8 - 24	8 - 24	8 - 24	8 - 24	8 - 24	8 - 24	8 - 24	8 - 24	8 - 24	16 - 24	16 - 24
Flux constant (µg/cm²/h)	0.0026	0.0038	0.0010	0.0024	0.0052	0.0049	0.0044	0.0034	0.0036	0.0022	0.0009	0.0031	0.0487	0.0298
Kp * 10^{-4} (cm/h)	0.0410	0.0609	0.0324	0.0803	0.0618	0.0772	0.1457	0.1134	0.0567	0.0350	0.0286	0.1037	47.5865	29.1384
Lag time (h)	0.9	0.1	0.0	3.9	1.6	0.0	0.0	1.0	0.0	0.0	0.0	0.0	11.3	11.4
R^2	0.9991	0.9995	0.9986	0.9938	0.9959	0.9914	0.9998	0.9984	0.9996	0.9994	0.9980	0.9992	0.9971	0.9983
Mean rel. absorption (% at 24h)	0.0364		0.0298		0.0372		0.0782		0.0289		0.0415		3.9152	
Mean abs. absorption (µg/cm²/24h)	0.0752		0.0370		0.1209		0.0928		0.0732		0.0531		0.6257	
Mean flux constant (µg/cm²/h)	0.0032		0.0017		0.0050		0.0039		0.0029		0.0020		0.0487	
Mean Kp * 10^{-4} (cm/h)	0.0509		0.0563		0.0795		0.1295		0.0459		0.0661		47.5865	
Mean lag time (h)	0.5		1.9		0.8		0.5		0.0		0.0		11.3	

D2: Data not included in calculations due to low recovery value.

Table VII Mean data of the cumulative absorption of arbutin and testosterone (Mean of experiments 1, 2 and 3)

	Cumulative absorption (µg/cm2)													
	A1		A2		B1		B2		C1		C2		D	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Mean cumulative absorption (µg/cm²)	0.0321	0.0411	0.0174	0.0176	0.0584	0.0577	0.0406	0.0428	0.0477	0.0282	0.0384	0.0292	0.4711	0.1745
Rel. absorption (% at 24h)	0.0154	0.0193	0.0153	0.0137	0.0203	0.0165	0.0361	0.0368	0.0195	0.0108	0.0339	0.0198	2.9278	1.0565
Flux constant (µg/cm²/h)	0.0016	0.0015	0.0009	0.0008	0.0025	0.0023	0.0019	0.0016	0.0022	0.0009	0.0017	0.0010	0.0300	0.0134
Kp * 10^{-4} (cm/h)	0.0256	0.0242	0.0295	0.0259	0.0403	0.0360	0.0625	0.0548	0.0355	0.0139	0.0553	0.0334	29.1783	12.9031
Lag time (h)	0.8	0.4	1.2	1.4	0.9	0.6	1.3	1.6	0.7	0.6	1.4	1.9	8.7	1.6

Appendix 3 Figures of the cumulative percutaneous absorption of arbutin

Figure 1 Cumulative percutaneous absorption of arbutin (Experiment 1)

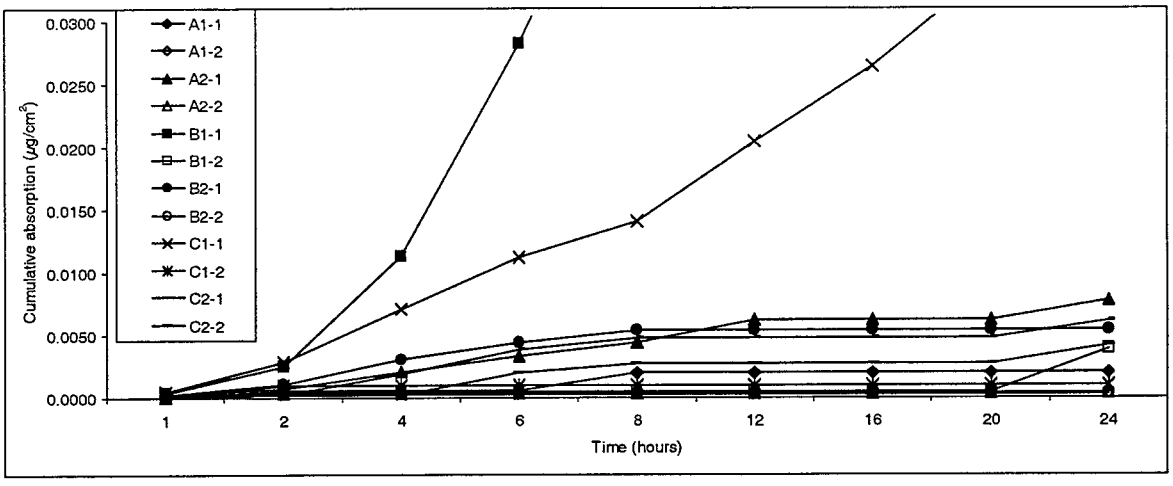
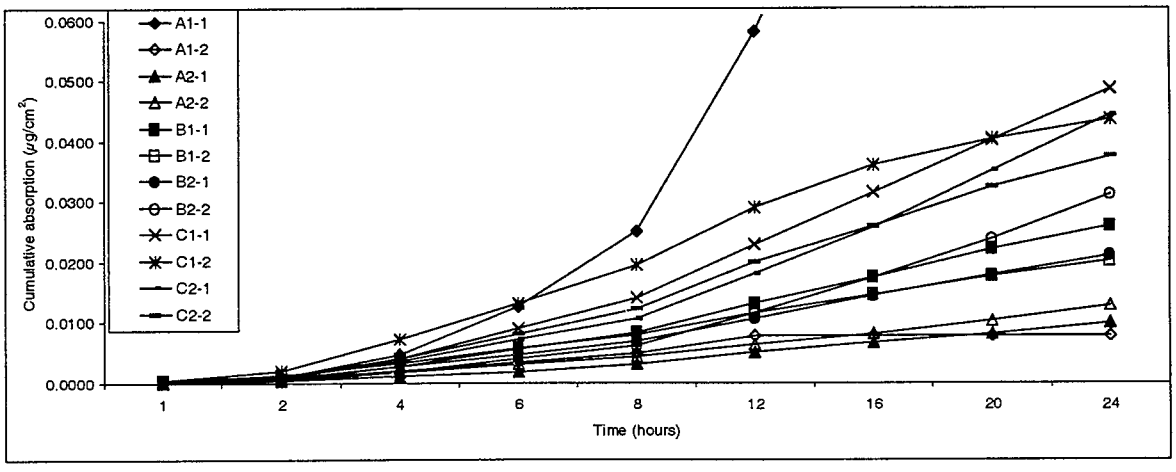
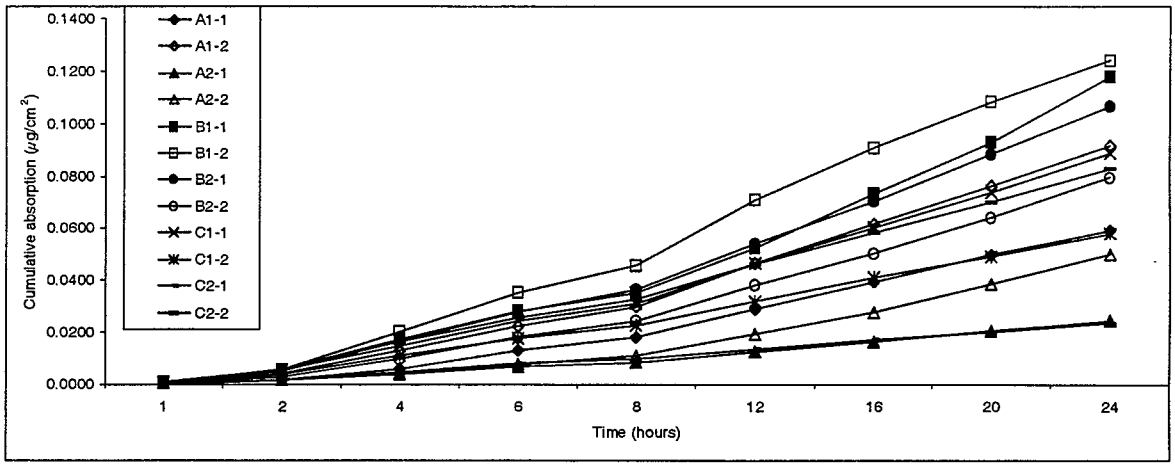


Figure 2 Cumulative percutaneous absorption of arbutin (Experiment 2)



Appendix 3 continued

Figure 3 Cumulative percutaneous absorption of arbutin (Experiment 3)



Appendix 4 Individual data of the tissue distribution of arbutin and testosterone

Table VIII Tissue distribution of arbutin and testosterone (Experiment 1)

Exp.1	Integrity Kp x 10^{-3} (cm/h)	Donor compartment (%)	Receptor fluid samples (%)	Receptor compartment (%)	Skin wash non-exposed area (%)	Skin wash exposed area (%)
A1-1	1.67	0.151	0.001	0.000	0.063	100.8
A1-2	1.28	4.597	0.000	0.000	0.151	97.3
A2-1	1.38	1.845	0.008	0.001	0.099	102.0
A2-2	1.33	1.306	0.000	0.001	0.253	102.7
B1-1	1.69	2.258	0.124	0.006	0.142	87.9
B1-2	0.62	0.161	0.002	0.000	0.236	98.5
B2-1	1.98	0.699	0.006	0.001	0.163	104.7
B2-2	0.54	0.819	0.000	0.000	0.079	98.3
C1-1	1.84	6.088	0.022	0.002	1.498	84.8
C1-2	0.91	0.744	0.001	0.001	0.451	110.0
C2-1	1.22	13.158	0.005	0.010	7.632	70.2
C2-2	0.84	16.690	0.004	0.001	0.965	93.1
D1	1.32	12.468	3.685	0.704	2.897	71.3
D2	1.40	8.914	3.258	0.645	2.101	68.3

B1-1: Data not included in calculations due to clearly deviating absorption profile.
C2-1: Data not included in calculations due to clearly deviating levels of test compound in the skin. Total absorption > than 3x mean value.

Exp. 1 (continued)	Tape strips (%) exposed area	Tape strips (%) non-exposed area	Epidermis (%)	Dermis (%)	Rest skin (%)	Total recovery (%)	Total absorption (%)
A1-1	0.052	0.002	0.007	0.005	0.003	101.1	0.016
A1-2	0.047	0.002	0.008	0.002	0.003	102.1	0.014
A2-1	0.139	0.001	0.036	0.005	0.004	104.2	0.053
A2-2	0.267	0.002	0.114	0.016	0.004	104.6	0.135
B1-1	0.353	0.002	0.113	0.057	0.016	91.0	0.316
B1-2	0.007	0.003	0.007	0.009	0.014	98.9	0.032
B2-1	0.422	0.003	0.069	0.029	0.011	106.1	0.116
B2-2	0.073	0.001	0.069	0.002	0.002	99.4	0.074
C1-1	0.728	0.010	0.089	0.062	0.033	93.3	0.209
C1-2	0.095	0.006	0.111	0.052	0.053	111.5	0.217
C2-1	0.667	0.009	0.130	0.585	0.471	92.8	1.202
C2-2	0.174	0.017	0.060	0.017	0.065	111.1	0.147
D1	1.796	0.084	1.564	3.210	1.622	99.3	10.786
D2	3.161	0.191	2.156	4.188	0.938	93.8	11.186

Total absorption: receptor fluid samples + receptor compartment + epidermis + dermis + rest skin

Appendix 4 continued

Table IX Tissue distribution of arbutin and testosterone (Experiment 2)

Exp.2	Integrity Kp x 10^{-3} (cm/h)	Donor compartment (%)	Receptor fluid samples (%)	Receptor compartment (%)	Skin wash non-exposed area (%)	Skin wash exposed area (%)
A1-1	0.57	0.230	0.058	0.006	0.329	87.9
A1-2	0.79	0.289	0.003	0.009	0.069	85.6
A2-1	0.75	0.439	0.013	0.005	0.332	93.1
A2-2	0.75	0.060	0.011	0.001	0.039	101.9
B1-1	0.69	0.098	0.012	0.002	0.073	101.6
B1-2	1.10	0.026	0.013	0.003	0.259	101.2
B2-1	0.84	4.178	0.019	0.002	0.106	99.7
B2-2	0.77	1.803	0.034	0.005	0.220	86.0
C1-1	0.70	3.969	0.019	0.005	0.378	85.8
C1-2	0.98	24.686	0.017	0.004	0.209	77.8
C2-1	0.79	2.727	0.044	0.009	0.053	90.4
C2-2	0.91	20.546	0.039	0.009	0.346	85.2
D1	0.77	15.809	1.319	0.310	1.159	70.9
D2	0.85	12.810	2.461	0.462	0.863	69.5

A1-1: Data not included in calculations due to clearly deviating absorption profile.

Exp. 2 (continued)	Tape strips (%) exposed area	Tape strips (%) non-exposed area	Epidermis (%)	Dermis (%)	Rest skin (%)	Total recovery (%)	Total absorption (%)
A1-1	1.058	0.028	0.920	0.092	0.031	90.7	1.107
A1-2	0.240	0.010	0.223	0.138	0.241	86.8	0.613
A2-1	0.358	0.012	0.105	0.077	0.024	94.5	0.224
A2-2	0.091	0.002	0.033	0.032	0.008	102.2	0.086
B1-1	0.201	0.009	0.059	0.054	0.010	102.2	0.137
B1-2	0.506	0.004	0.209	0.032	0.009	102.3	0.266
B2-1	0.291	0.004	0.073	0.059	0.013	104.4	0.166
B2-2	0.395	0.005	0.154	0.069	0.017	88.7	0.279
C1-1	0.110	0.006	0.013	0.041	0.021	90.4	0.099
C1-2	0.101	0.004	0.010	0.016	0.011	102.9	0.059
C2-1	0.086	0.002	0.030	0.056	0.026	93.4	0.165
C2-2	0.098	0.007	0.011	0.089	0.037	106.4	0.185
D1	2.994	0.154	1.040	6.220	1.088	101.0	9.977
D2	2.275	0.159	1.595	8.228	1.418	99.8	14.164

Total absorption: receptor fluid samples + receptor compartment + epidermis + dermis + rest skin

Appendix 4 continued

Table X Tissue distribution of arbutin and testosterone (Experiment 3)

Exp.3	Integrity Kp x 10^{-3} (cm/h)	Donor compartment (%)	Receptor fluid samples (%)	Receptor compartment (%)	Skin wash non-exposed area (%)	Skin wash exposed area (%)
A1-1	0.76	0.009	0.039	0.001	0.149	96.8
A1-2	1.09	4.162	0.034	0.001	0.111	89.0
A2-1	0.96	0.246	0.019	0.001	0.107	102.3
A2-2	1.12	1.513	0.040	0.002	0.142	94.6
B1-1	0.95	0.709	0.032	0.003	0.095	98.6
B1-2	0.87	8.041	0.042	0.002	0.197	86.4
B2-1	1.20	1.158	0.064	0.003	0.125	89.7
B2-2	0.81	9.587	0.093	0.004	0.282	92.9
C1-1	0.91	7.584	0.033	0.003	0.171	88.8
C1-2	0.98	6.372	0.025	0.001	0.247	87.6
C2-1	0.89	1.648	0.026	0.002	0.478	88.8
C2-2	0.96	10.356	0.057	0.004	0.476	84.0
D1	0.86	7.931	3.915	0.384	2.376	68.7
D2	0.72	7.891	2.373	0.219	1.579	54.1

D2: Data not included in calculations due to low recovery value.

Exp. 3 (continued)	Tape strips (%) exposed area	Tape strips (%) non-exposed area	Epidermis (%)	Dermis (%)	Rest skin (%)	Total recovery (%)	Total absorption (%)
A1-1	0.012	0.002	0.012	0.010	0.006	97.1	0.068
A1-2	0.007	0.098	0.033	0.015	0.006	93.5	0.090
A2-1	0.009	0.008	0.046	0.016	0.021	102.8	0.104
A2-2	0.064	0.007	0.087	0.018	0.007	96.5	0.155
B1-1	0.050	0.001	0.042	0.037	0.022	99.6	0.136
B1-2	0.046	0.004	0.068	0.017	0.014	94.9	0.142
B2-1	0.015	0.007	0.228	0.064	0.016	91.4	0.375
B2-2	0.106	0.008	0.064	0.078	0.032	103.2	0.271
C1-1	0.052	0.004	0.042	0.029	0.033	96.8	0.139
C1-2	0.038	0.003	0.035	0.006	0.016	94.4	0.084
C2-1	0.082	0.007	0.052	0.025	0.045	91.1	0.151
C2-2	0.089	0.003	0.057	0.032	0.025	95.1	0.175
D1	0.643	0.205	4.723	4.161	1.649	94.7	14.833
D2	0.651	0.178	2.605	2.475	1.695	73.8	9.367

Total absorption: receptor fluid samples + receptor compartment + epidermis + dermis + rest skin

Appendix 4 continued

Table XI Tissue distribution of arbutin and testosterone (mean of experiments 1, 2 and 3)

Mean		Integrity Kp x 10^{-3} (cm/h)	Donor compartment (%)	Receptor fluid samples (%)	Receptor compartment (%)	Skin wash non-exposed area (%)	Skin wash exposed area (%)
A1	Mean	1.12	1.842	0.015	0.002	0.109	93.9
	SD	0.38	2.324	0.019	0.004	0.042	6.3
A2	Mean	1.05	0.901	0.015	0.002	0.162	99.4
	SD	0.28	0.745	0.014	0.002	0.109	4.4
B1	Mean	0.85	1.807	0.020	0.002	0.172	97.3
	SD	0.19	3.496	0.016	0.001	0.084	6.2
B2	Mean	1.02	3.041	0.036	0.003	0.162	95.2
	SD	0.51	3.454	0.036	0.002	0.076	6.9
C1	Mean	1.05	8.240	0.020	0.003	0.493	89.1
	SD	0.40	8.408	0.011	0.002	0.504	10.9
C2	Mean	0.88	10.394	0.034	0.005	0.464	88.3
	SD	0.07	8.336	0.020	0.004	0.330	3.7
D	Mean	1.04	11.587	2.928	0.501	1.879	69.7
	SD	0.30	3.186	1.056	0.169	0.849	1.3

Mean (continued)		Tape strips (%) exposed area	Tape strips (%) non-exposed area	Epidermis (%)	Dermis (%)	Rest skin (%)	Total recovery (%)	Total absorption (%)
A1	Mean	0.071	0.023	0.057	0.034	0.052	96.1	0.160
	SD	0.096	0.042	0.093	0.058	0.106	6.2	0.255
A2	Mean	0.154	0.005	0.070	0.027	0.011	100.8	0.126
	SD	0.132	0.004	0.036	0.026	0.009	4.3	0.060
B1	Mean	0.162	0.004	0.077	0.029	0.014	99.6	0.143
	SD	0.206	0.003	0.077	0.018	0.005	3.0	0.083
B2	Mean	0.217	0.005	0.109	0.050	0.015	98.9	0.214
	SD	0.175	0.003	0.067	0.029	0.010	7.2	0.114
C1	Mean	0.187	0.006	0.050	0.034	0.028	98.2	0.135
	SD	0.266	0.003	0.041	0.021	0.015	7.7	0.066
C2	Mean	0.106	0.007	0.042	0.044	0.040	99.4	0.164
	SD	0.039	0.006	0.021	0.029	0.016	8.8	0.016
D	Mean	2.174	0.159	2.216	5.201	1.343	97.7	12.189
	SD	1.018	0.047	1.456	2.017	0.319	3.2	2.166

Total absorption: receptor fluid samples + receptor compartment + epidermis + dermis + rest skin

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TNO report

V6035

Skin metabolism after repeated topical application of Arbutin in human volunteers

Date	June 30, 2005
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Sponsor	Shiseido Co. Ltd, 2-12-1, Fukuura, Kanazawa-ku, Yokohama-shi, Kanagawa, 236-8643, Japan
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Number of pages	42
Number of tables	10
Number of figures	1
Number of appendices	15

Contents

Reason for revised final report

To have consistent Hydroquinone and Arbutin skin biopsy results throughout the final report a revised final report has been drafted. All hydroquinone skin biopsy results have been reported now as < 1.1 ng per skin biopsy rather then < 22 ng which is the result taken relative to the mean dismembrated weight. Corrections were made on page 8, 9, 27, 28 (Table 2; foot note), 38 and 39. Also appendix 14.1.7.1 has been changed accordingly.

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DECLARATION AND SIGNATURE PAGES

AUTHENTICATION BY THE PRINCIPAL INVESTIGATOR

TNO Quality of Life

I, the undersigned, hereby declare that to the best of my knowledge this report constitutes a true and complete representation of the procedures followed and of the results obtained in this study by TNO Quality of Life. The study was carried out under my overall supervision and conducted in accordance with the ICH Guideline for Good Clinical Practice (ICH topic E6, adopted 01-05 1996 and implemented 17-01-1997).

W.J.A. Meuling, BSc

Principal Investigator

30/06/05

Date / Signature

Approved by

Ms. A.F.M. Kardinaal, PhD

Head Operations Business unit Physiological Sciences

30/06/05

Date / Signature

STATEMENT BY THE MEDICAL INVESTIGATOR

I, the undersigned, hereby declare that to the best of my knowledge the clinical data presented in this report were compiled under my supervision, and accurately reflect the data obtained[1]

L. Kok, MD, PhD[1]

Medical Investigator

30/6/05
Date / Signature

STATEMENT BY THE STATISTICIAN

I, the undersigned, hereby declare that to the best of my knowledge the statistical data presented in this report were compiled by me or under my supervision, and accurately reflect the data obtained.

C. Kistemaker, BSc

Statistician

30-06-05
Date / Signature

[1] Mrs L. Kok has left the TNO organisation as of the date of issue of this Revised Final version. Her responsibilities and duties were taken over by Mrs M. Klever, M.D.

Synopsis

Name of company: Shiseido CO Ltd. **Name of finished product:** CP-SEN **Name of active substance(s):** Arbutin	**INDIVIDUAL TRIAL TABULAR FORMAT** **Referring to Part of the dossier** **Volume:** **Page:**	**(For National Authority use only)**
Title of the trial:	Skin metabolism after repeated topical application of Arbutin in human volunteers	
Investigators:	**Principal Investigator**: W.J.A Meuling, BSc **Medical Investigator**: L. Kok, MD, PhD **Statistician**: C. Kistemaker, BSc	
Study center:	TNO Quality of Life, Business unit Physiological Sciences, P.O. Box 360, NL-3700 AJ Zeist, the Netherlands, Phone.: +31-30 694 41 44, Fax: +31-30 695 72 24, Visitors address: Utrechtseweg 48, Zeist, the Netherlands	
Study period:	FSI 10 JAN 2005 LSO 14 JAN 2005	Clinical Phase: N.A.
Objectives:	The primary study objective is to establish skin metabolism of the active ingredient (Arbutin) present in a repeated topically applied formulation.	
Methodology/Design:	A multiple (topically) dosed, open study	
Number of subjects:	Eighteen (18) apparently healthy female (9) and male (9) volunteers participated in one study group	
Diagnosis and main criteria for inclusion:	Only healthy subjects according to set in- and exclusion criteria, pre-study check-up results, physical examination results, participated in this study	
Test product, dose, mode of administration, batch No:	Repeated topical application onto an area of 50 cm^2 of a formulation containing 6.3% (w/w) Arbutin.	
Study restrictions	**Diet**: Not applicable. Subjects kept a diary of their daily food and drink intake. **Cosmetics**: No use of hair dyes during the study and three days prior to Day 01 of the study **Bathing:** During the whole topical treatment period, showering and bathing was only allowed in the morning just prior to visiting TNO. Swimming, sauna visit and use of sunbeds during the whole topical application period were <u>not allowed.</u> **Clothing:** A daily dispatched g-string not covering the applicated area was worn under the usually daily clothing.	
Reference therapy, dose, mode of administration, batch No:	Not applicable.	
Duration of treatment:	Repeated topical application on 4 consecutive days	
Criteria for evaluation:	**Primarily:** The Arbutin and hydroquinone content in three combined skin biopsies derived either from a control or a repeatedly treated skin area. **Secondary:** Urinary hydroquinone amounts corrected for diuresis by creatinine content.	

Name of company: Shiseido CO Ltd. **Name of finished product:** CP-SEN **Name of active substance(s):** Arbutin	**INDIVIDUAL TRIAL TABULAR FORMAT** **Referring to Part of the dossier** **Volume:** **Page:**	**(For National Authority use only)**

Statistical methods:
Comparisons between individual treatment means of the study parameters (Arbutin and hydroquinone content of biopsies) were evaluated using a 2-sided paired Student t-test at a probability level ($p \leq 0.05$). Demographics and anthropometrics are reported descriptively and tabulated.

SUMMARY – CONCLUSIONS:

This report describes the conduct and the results of a human volunteer study with topical treatment of a gel containing Arbutin (CP-SEN). The primary study objective was to establish skin metabolism of Arbutin to hydroquinone after repeated application of an Arbutin containing formulation. The skin metabolism was evidenced by Arbutin and hydroquinone amounts established in skin biopsies obtained from a treated area (right buttock) of 50 cm^2 after repeated application (140.1 mg) for 4 days and compared to skin biopsies taken from an untreated contralateral (control) site (left buttock).

In all control biopsies low levels (<1.1 ng) of hydroquinone per skin biopsy were established. This indicates that the actual concentration of hydroquinone in untreated human skin is very low. Higher levels of hydroquinone, on average 177 ± 149 ng/g (range: 32.0 – 602 ng/g) were found in the biopsies derived from the treated skin area when taken relative to the dismembrated weight. Furthermore, it was observed that for all control skin biopsies collected on Day 01 of the study Arbutin levels were below 8.9 ng, except for one. This leads to the conclusion that Arbutin concentrations in human skin is also low.

In 5 out of 18 treated samples elevated levels of Arbutin could be established, the lowest level (863 ng/g) was established in subject 04 while the highest level (9809 ng/g) was found in subject 17. On average, 3736 ng/g ± 2138 (range: 863-9809 ng/g) was established in these samples. When the hydroquinone content in these samples is taken relative, on a weight to weight basis, to the Arbutin+hydroquinone content, on average 4.6% (± 2.9) (range: 1.69-11.77) of hydroquinone is present in these skin samples. Furthermore, the actual (low) levels of hydroquinone established in treated skin (µg/cm^2) amounted, on average, to 0.018 ± 0.016 (µg/cm^2), range 0.003 – 0.072 (µg/cm^2).

Differences between the skin data results of Day 01 and Day 05 for arbutin and hydroquinone were statistically analysed. The statistical tests showed significant differences between Day 01 and Day 05 for all variables ($p<.0001$), except for the variable: weight dismembrated ($p=0.5872$) for Arbutin and Hydroquinone, since this was derived from the same sample.

It is well known that food contains hydroquinone levels and thus consequently, also the human body contains certain levels of hydroquinone. The latter is evidenced by urinary HQ levels. Therefore, spot urine samples were collected in the study on a daily basis just prior to topical application to establish possible changes in urinary HQ levels.

In 24 out of 90 urine samples a concentration of (total) hydroquinone above the lowest calibration point (0.974 mg/l) could be established. It was observed that on Day 01, 6/18 samples, Day 02, 6/18 samples, Day 03, 8/18 samples and on Day 04 and Day 05, 2/18 samples revealed (total) hydroquinone levels above the LOQ. The collected spot urine samples were corrected for diuresis by the respective creatinine content of the sample. The highest hydroquinone/creatinine ratio (9.16 mmol/mol) was observed on Day 02 (subject 04). Furthermore, subject 04 showed also the highest levels on Day 01 (8.45 mmol/mol) and Day 05 (8.82 mmol/mol). However, based on the large variation in urinary total HQ results, changes in urinary HQ levels due to topical treatment of Arbutin could not be established.

Efficacy results
Efficacy of Arbutin (CP-SEN) has not been an objective in this study

Name of company: Shiseido CO Ltd. **Name of finished product:** CP-SEN **Name of active substance(s):** Arbutin	**INDIVIDUAL TRIAL TABULAR FORMAT** **Referring to Part of the dossier** **Volume:** **Page:**	**(For National Authority use only)**

Safety results

All participants reported their well-being. As was judged by the medical investigator, no significant clinically relevant laboratory pre-study check up results were reported. All participants experienced complaints (redness of skin) after the first set of biopsies collected on Day 01, which was reported definitively related to the study design. All other complaints were minor and reported not or unlikely related to the study treatment. Finally all subjects left the study without complaints, signs or symptoms of adverse (systemic) effects due to or related to the repeated topical application of CP-SEN gel containing Arbutin.

Conclusions

The following conclusions can be drawn from this study:

- In all control skin biopsy samples very low levels of hydroquinone (< 1.1 ng) and Arbutin (< 8.9 ng) per skin biopsy were present;
- In all treated skin samples hydroquinone, on average 177 ± 149 ng/g (range: 32.0 – 602 ng/g) and Arbutin, on average, 3736 ng/g ± 2138 (range: 863-9809 ng/g) when taken relative to the dismembrated weight could be established;
- Repeated topical application of CP-SEN gel containing 6.3% (w/w) Arbutin leads to detectable amounts of Arbutin and hydroquinone in skin;
- The statistical tests showed significant differences ($p < .0001$) between Day 01 and Day 05 for the variables: analysed amount (ng) and corrected amount (ng/g) for hydroquinone as well as for Arbutin;
- In a number of spot urine samples (24/90) detectable hydroquinone levels, corrected by creatinine for diuresis (range: < 4.19 – 9.16 mmol/mol), were established;
- Based on the large variation in the established urinary total HQ results, changes in urinary HQ levels due to topical treatment of Arbutin could not be established;
- When the hydroquinone content in the skin samples is taken relative, on a weight to weight basis, to the Arbutin+hydroquinone content, on average 4.6% (± 2.9) (range: 1.69-11.77) of hydroquinone is present in these skin samples.
- Actual levels of hydroquinone in treated skin amounted on average to 0.018 ± 0.016 µg/cm^2, range 0.003 – 0.072;
- Repeated topical application of CP-SEN gel containing 6.3% (w/w) Arbutin was well tolerated by all subjects in this study.

Date of report

30 June 2005

1 List of abbreviations (and definitions of terms)

AE	:	Adverse Event
Arb	:	Arbutin
b.w.	:	body weight
CAL	:	Calibration sample
CAS (nr.)	:	Chemical Abstract Services (number)
CCMO	:	Central Committee on Research involving Human Subjects
CRO	:	Contract Research Organisation
EC	:	European Community
GC-MS	:	Gaschromatograph Mass Spectrometry
GCP	:	Good Clinical Practice
GLP	:	Good Laboratory Practice
HQ	:	Hydroquinone
ICH	:	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
LC-ECD	:	Liquid Chromatograph- Electron Chemical Detection
METC	:	Medisch Ethische ToetsingsCommissie (Medical Ethics Committee
MOS	:	Margin of Safety
NOAEL	:	No Observed Adverse Effect Level
OECD	:	Organisation for Economic Cooperation and Development
QAU	:	Quality Assurance Unit
QC	:	Quality Control
SAE	:	Serious Adverse Event
SAR	:	Statistical Analysis Report
SCCP	:	Scientific Committee on Consumer Products
SOP	:	Standard Operating Procedure
TNO	:	Nederlandse organisatie voor Toegepast Natuurwetenschappelijk onderzoek (Netherlands Organisation for Applied Scientific Research)
UMC-U	:	University Medical Center - Utrecht
VTC	:	Visit Time Code
WBC	:	White Blood Cell
WMA	:	World Medical Association
WMO	:	Wet Medisch Onderzoek met mensen (Medical Research involving Human Subjects Act)

2 Ethics

2.1 Independent Ethics Committee

The study protocol has been drafted in accordance with the current ICH Guideline for Good Clinical Practice (ICH Topic E6, Guideline for Good Clinical Practice, adopted 01-05-1996 and implemented 17-01-1997).
The protocol and Amendments 01, 02 and 03 to protocol were submitted to the Medical Ethics Committee (METC-U) and approval had been given on 02 November 2004, 04 January 2005, 11 January 2005, and 05 April 2005, respectively.

2.2 Ethical conduct of the study

The study was conducted according to:

1. The current revision (52nd) of the World Medical Association General Assembly, Declaration of Helsinki (Edinburgh, Scotland, October 2000), and the Note of clarification on paragraph 29 added at the WMA General Assembly, Washington, USA, October 2002;
2. The ICH Guidelines for Good Clinical Practice (ICH Topic E6, adopted 01-05-1996 and implemented 17-01-1997);
3. The Dutch Medical Research involving human Subjects Act („Wet medisch wetenschappelijk onderzoek met mensen“, WMO, 01-12-1999);
4. The current national regulations.

2.3 Subject information and consent

Thirty six (36) positively responding potential candidates were invited to come to TNO for an oral briefing in the presence of several members of the project team during which they were informed about the aim, the procedures, the constraints, the insurance cover and the financial compensation of the study. Two oral briefings, a morning and an afternoon session, were held on 25 November 2004. Prior to this meeting, all potential candidates received a copy of the information package ‘Schriftelijke informatie proefpersonen’ (P6035 B01; Appendix 14.1.1.), that fully covered the information that was actually given verbally during the meeting. After the respondents became familiar with the content and procedures of the study, those who were interested to participate undersigned, in duplicate, the informed consent form (P6035 F01 in Dutch; appendix 14.1.4), one of which they retained. Finally, 28 potential candidates were subjected to a pre-screening including: clinical laboratory tests, a study specific physical examination and an anamnese based on a completed health and lifestyle questionnaire. At last, the medical investigator, based on the clinical check up results, established the eligibility of twenty one (21) subjects for the study. Three subjects were assigned substitutes. Due to not having validated analytical methods in time the clinical part of the study was postponed from 20 December 2004 till 10 January 2005 (Day 01). Prior to the start on Day 01 of the study all subjects received an updated subject information package dated 04 January 2005 (P6035 B01; Appendix 14.1.1; Amendment 01) and undersigned, in duplicate an updated informed consent (P6035 F01 in Dutch, appendix 14.1.4).

3 Investigators and study administrative structure

The sponsor was responsible for the financial compensation for the conduct of the study. The sponsor was liable for the study formulations, for the prompt delivery to TNO and was responsible for arranging and delivery of detailed information regarding the description of the test formulations including a certificate of analysis. Moreover, the sponsor was responsible for the insurance according to the "WMO" (Dutch law). However, at their request the responsibility was taken over by TNO Quality of Life. Insurances for material damage and accidents during travel to and from TNO and during the stay at TNO were also the responsibility of TNO.

W.J.A. Meuling, BSc was responsible for the overall conduct of the study, for drafting the protocol and the interim - and final report.

Ms. L. Kok, MD, PhD was responsible for the safety of the subjects, the medical aspects of the study, the documentation, and interpretation and reporting of possible AEs and SAEs. A part of the screening has been delegated to an assistant medical investigator appointed by the management.

The actual skin biopsy collection was the responsibility of either J.N. Bennen, MSc, MD, (Day 01) or S. Pavel, MD, PhD, PhD, (Day 05), both Board Certified Dermatologists.

F.W. Sieling was responsible for the daily conduct of the clinical part of the study and the contacts with the subjects. The direct involvement in the daily conduct of the clinical part was delegated to J.A.M. Jacobs, study nurse.

R.A.Woutersen, PhD was as head of the Business unit Toxicology Applied Pharmacology the overall responsible person for the clinical chemistry and haematology analysis in blood and urine in the pre-study screen and for the creatinine content of the in study urine samples The actual analysis has been delegated to Mr. J.F. Catsburg.

L.P. Brüll, PhD, co-investigator of the TNO Business unit Analytical Sciences was responsible for the analysis of Arbutin and hydroquinone in the plasma, urine and the skin biopsies samples. The chemical analysis has been carried out according to the Principles of Good Laboratory Practice (GLP).

Testing facility

The study has been conducted by:

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Retention of samples and records

The following documents will be retained in the archives of TNO Quality of Life, Location Zeist, Utrechtseweg 48, 3704 HE Zeist, The Netherlands, during 15 years after the report of the study has been issued:

1. Master copies of the approved study protocol, and final report
2. All documents containing personal data of individual trial subjects
3. Raw data (source documents or authenticated copies of these) of analyses conducted at TNO Quality of Life.
4. Correspondence
5. All other information related to tests and analyses conducted at TNO Quality of Life

The following samples and specimens will be retained in appropriate facilities of TNO Quality of Life:

1. A representative part of the study substance which will be retained for 5 years
2. The remainder of the in-study urine samples will be retained for three months after the final report has been approved by the sponsor. All remaining samples will be discarded, after the periods given, at the investigators site.

4 Introduction

There are individuals with pigment spots or hyper-pigmentations due to increased melanin production such as age spots, freckles, sun spots, pimples, etc. Although these spots in most cases do not have a direct clinical relevance, from a cosmetic point of view these spots are sometimes not acceptable by individuals. Therefore, so-called skin whitening products are currently available on the market. These products claim to be helpful in reducing these type of spots since they help to slow down melanin production and giving your skin a more even look over time. Among others CP-SEN-gel is such a skin whitening product which contains Arbutin (hydroquinone-ß-D-glucopyranoside) as the active ingredient. After skin absorption Arbutin may be metabolised to hydroquinone by the enzym ß-glucosidase. Hydroquinone has been reported to be a melanin blocker. However, hydroquinone has also been reported to be a skin irritant at elevated concentrations (>5-10%). Although, hydroquinone is not present in the CP-SEN-gel under investigation, to date reliable safety data on formed hydroquinone in the skin with respect to repeated topical use of the Arbutin containing gel was not available. It is well known that all kinds of food contains levels of hydroquinone, therefore it is obvious that levels of hydroquinone could be present in the body which can be evidenced by collecting urine.
In the present study the skin metabolism of Arbutin present in CP-SEN-gel and applied to the skin daily on four consecutive days has been investigated by skin biopsy collected at pre-determined time points analysed for Arbutin and hydroquinone content.

The present study protocol has been drafted in accordance with, and the study has been conducted according to the ICH Guideline for Good Clinical Practice (ICH Topic E6; Guideline for Good Clinical Practice) adopted 01-05-1996 and implemented 17-01-1997.

5 Study objective

The primary study objective was to investigate skin metabolism to hydroquinone of the active ingredient (Arbutin) present in a repeated topically applied formulation.
The secondary study objective was to investigate urinary concentrations of hydroquinone prior to daily topical application or to biopsy.

6 Investigational plan

The investigational plan has been outlined in detail in Protocol P6035, dated 22 November 2004, version revised final 1 and Amendment 1, 2 and 3 to Protocol, dated 04 January 2005, 06 January 2005 and 05 April 2005, respectively (see Appendix 14.1.1). The protocol will be mentioned in this report further as 'Protocol P6035'.

6.1 Overall study design and plan description

The study has been designed as a multiple (topical) dosing, open study.

6.2 Discussion of study design and choice of control group

In this study no control group has been used.

6.3 Selection of study population

The subjects in this study were recruited from the pool of volunteers of TNO Quality of Life, Location Zeist, Utrechtseweg 48, 3704 HE Zeist, The Netherlands in compliance with the following in- and exclusion criteria:

Inclusion criteria
1: Age 18-45 years at Day 01 of the study (Gender: female/male)
2: Healthy as assessed by health and lifestyle questionnaire, skin inspection and clinical laboratory results
3: Voluntary participation
4: Having given their written informed consent
5: Non smoker
6: Willing to comply with the study procedures
7: Willing to refrain from using hair dyes during the study and three days in advance of Day 01.
8: Willing to accept use of all anonymized data, including publication, and the confidential use and storage of all data
9: Willing to accept the disclosure of the financial benefit of participation in the study to the authorities concerned

Exclusion criteria
Subjects with one or more of the following characteristics were excluded from participation:
1: Participation in any clinical trial including blood sampling and/or administration of substances up to 90 days before Day 01 of this study
2: Prescribed medication (except paracetamol and oral contraceptives)
3: Alcohol consumption more than 21 units/week (1 unit of alcohol equals 10 grams of ethanol)
4: Recent blood donation (<1 month prior to Day 01 of the study)
5: TNO personnel and their relatives in the first and second remove
6: Having a history of medical or surgical events that may significantly affect the study outcome including dermatological diseases such as having dermatitis or particular skin diseases
7: Having scars, cuts, wounds, dermal abnormalities in the testing areas
8: Not having a general practitioner
9: Not willing to accept information-transfer concerning participation in the study, or information regarding his health, like laboratory results, findings at anamnesis or physical examination and eventual adverse events to and from his general practitioner

6.4 Study restrictions

Diet: Not applicable. Subjects has to keep a diary of their daily food and drink intake.
Cosmetics: No use of hair dyes during the study and three days prior to Day 01 of the study
Bathing: During the whole topical treatment period, showering and bathing was only allowed in the morning just prior to visiting TNO.
Swimming, sauna visit and using sun beds during the whole topical application period was not allowed.
Clothing: A daily dispatched g-string not covering the applicated area, has to be worn under the usually daily clothing.

6.5 Treatment

Subjects were treated for 4 consecutive days with an Arbutin containing gel (CP-SEN) onto an area of 50 cm^2 at the right side of the buttock.

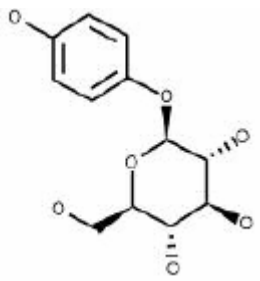
6.6 Identity of investigational products

Description of the study formulation

- Name (trivial) : CP-SEN
- Galenic form : Gel
- Aim : Skin whitening
- Batch number : 043
- Expiry date : 12 October 2007
- Storage conditions : Ambient (dark)
- Received (date) : 06 december 2004
- Number of vials : 25
- TNO dispense number : 04015F

Description of the active ingredient (Arbutin)

- Active ingredient (a.i) : Arbutin (6.3% w/w)
- Chemical name : Hydroquinone-ß-D-glucopyranoside
- Empirical formula : $C_{12}H_{16}O_7$
- Structure formule



- Molecular weight : 272.25
- CAS number a.i. : 497-76-7
- Solubility (water) : Good

Relevant toxicicological data:
NOAEL
(13-week rat dermal study) : 618 mg/kg b.w./day

Skin irritation (rabbit/human) : Not irritating
Skin sensitization (guinea pig) : Not sensitizing (Magnusson-Kligman max. test)

The preparation of the study formulation has been carried out by the sponsor.
Also the analyses for the identity, the quality and purity of the batch of the study formulation has been conducted by the sponsor. A certificate of analysis and a product specification signed by an authorized person has been provided to TNO by the sponsor and is given in Appendix 14.1.2.

All documents with regard to the test substances used are archived at TNO Quality of Life, Location Zeist, Utrechtseweg 48, 3704 HE Zeist, The Netherlands.

6.6.1 Study approach
In this study repeated topical application of Arbutin formulation on 4 consecutive days has been carried out. To establish skin metabolism, skin biopsies were obtained from each subject. Three biopsies from a non-treated area (control) on Day 01 and 3 biopsies from the treated area were obtained on day 05 (about 24h-30h post application on day 04). The 3 biopsies obtained on the respective study day were combined to one sample. These pooled samples were analysed for their Arbutin and hydroquinone content and compared.

6.6.2 Method of assigning subjects to treatment
All subjects were allocated to an entry number randomly based on their order of arrival on Day 01 of the study. Entry numbers consisted of the TNO study code (6035), followed by a slash ('/'), followed by a 2-digit number starting with 01.

6.6.3 Selection of doses in the study
The selection of the topical application formulation dose of about 2.8 mg/cm^2 has been based on the general accepted dermal dose for cosmetics by the Scientific Committee on Consumer Products (SCCP).

6.6.4 Selection and timing of dose for each subject
At the scheduled study days (Day 01, Day 02, Day 03 and Day 04) subjects visited TNO and topical application of CP-SEN gel was performed. Prior to the start of biopsy collection (control) on Day 01 and biopsy collection on Day 05, and daily application on Day 02-Day 04, all subjects provided an urine sample.

6.6.5 Pre-treatment preparatory activities
To be able to apply controlled amounts of CP-SEN gel in the study two positive displacement pipettes (Transferpettor 100 µl, Brand, Germany) were calibrated in advance. Amounts of CP-SEN gel were taken ten fold and weighed. It was observed that when the displacement pipettes were set at 72 µl, this resulted, on average, to 70.03 ± 0.41 mg of CP-SEN gel application. Or when this amount is applied two times, to 140.1 ± 0.83 mg. Also the adherence of CP-SEN gel to latex handgloves after spreading out using circular motion was investigated in five fold. This resulted in low (<1.8 ± 1.1 mg) adherance. The latter has been neglected and was not taken into account in the study.

6.6.6 Topical treatment
All subjects were treated four times with the study formulation (CP-SEN-gel). Application was done within a delineated area of 50 cm^2 (10 x 5 cm) on one side of the upper part of the buttock (right side). The topical applications were carried out daily on four consecutive days onto the same area.
Subjects were applicated with CP-SEN-gel containing 6.3% (w/w) Arbutin. Individual coded glass vials with study formulation were used to topically apply small evenly-spaced

blobs within the test areas with a calibrated positive displacement pipet. The product has been applied by nurses, spread-out evenly by the index finger covered with a disposable glove using circular motions until the product has blended with the skin. For each subject and each application another new glove was used.
Day 01: the topical application period started on Day 01 and continues till Day 04. Per subject, 140.1 mg formulation per skin area has been applied daily, which corresponds to about 2.8 mg/cm^2 of formulation. This resulted in a daily application of ~8.9 mg of Arbutin. After finishing each application the subjects were instructed to leave open (not occluded) the applicated area for 60 minutes and were therefore confined to the TNO facility. To avoid direct contact with underwear with the applicated area subjects wore specific underwear (g-string) for the rest of the day under their daily clothing.
See figure 1 for details.

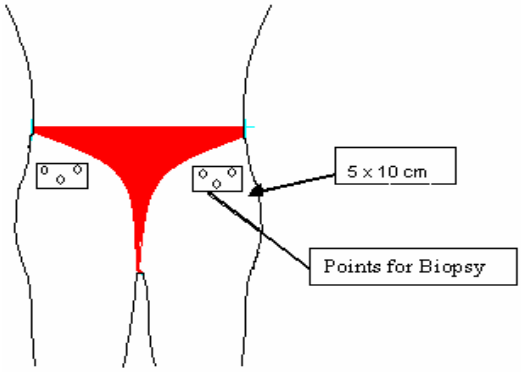


Figure1. Drawing to visualise the topical application site and the sites for punch biopsies

The next day, study Day 02, subjects arrived at TNO after having had a shower or a bath with soap or soap products at home, undressed themselves sofar as appropriate, whereafter a renewed topical application of the formulation was carried out and the same procedure as described above was repeated, for the consecutives study days (Day 03-04), the same procedure was followed.
On Day 05 they arrived at TNO after having had their bath or shower at home, undress themselves sofar as appropriate, whereafter a part of the repeatedly applicated area was tape stripped and disinfected prior to biopsy.

6.6.7 Skin stripping
Just prior to skin biopsy on Day 01 (control) the disinfected area was firstly skin stripped to remove the stratum corneum. Therefore, strips of adhesive cellophane tape (3M Company Scotch® Magic®, 19 mm) were used. Sixteen tape (16) strips in total were applied, one after the other with gentle pressure to the untreated (control) area; each was stripped off in a few seconds in a slow evenly manner. The strips were discarded.
On Day 05 the same procedure as on Day 01 was followed for the treated area.

6.6.8 Biopsy collection
On Day 01 prior to the first application three control skin biopsies were obtained from an untreated skin stripped area at the upper part of the left buttock. After four consecutive daily applications at approximately 24-30 hours following the last application (Day 04) also three skin biopsies were taken from the treated area at the contralateral upper part of the buttock. The collected (3) biopsies on each study day (Day 01 and Day 05), were combined and transferred to a pre-weighed coded container, weighed, recorded, sealed and placed in liquid nitrogen to get frozen immediately and stored refrigerated at -70°C awaiting pre-preparation and analysis.

Skin biopsies were taken according to the following procedure:

Day 01: The control test area after skin stripping was disinfected by a solution of 70% (w/w) ethanol. Then the biopsy site (10 x 2 cm) was sprayed with Cool spray® by the dermatologist and three punch biopsies (Stiefel; 4 mm) were taken from this area.
On Day 05 a slightly altered method has been used.
Day 05: After skin stripping, the treated test area was disinfected by a solution of 70% (w/w) ethanol. Then the biopsy site (3 x 2 cm) was sprayed within a rubber cone with ChloorEthyl spray® by the dermatologist and three punch biopsies (Stiefel; 4mm) were taken within this area. Thereafter, on both study days the little wounds were covered by a sterile bandage with hemostatic sponges.
The whole procedure has been carried out by board registered dermatologists.

6.6.9 Food intake and drink questionnaire
To record their daily food and drink intake subjects were instructed to keep a daily (Day 01-Day 05) diary (Appendix 14.1.1.; P6035 F06). Diaries were collected on a daily basis at every visit to TNO. Appendix 14.2.5 gives the collected diaries (in Dutch).

6.6.10 Blinding
Blinding was not applicable since the study was an open study.

6.6.11 Prior and concomitant therapy / treatment
At each visit to TNO subject's intake of medication as well as prescribed medication or so-called over-the counter drugs were recorded (Well-being questionnaire).

6.6.12 Treatment compliance measurement
The repeated topical application in each subject was carried out daily by nurses using calibrated positive displacement pipettes and recorded on forms at the respective study Day. No other treatment compliance check took place thereafter.

6.7 Efficacy and safety variables

6.7.1 Efficacy and safety measurements assessed
No efficacy measurements were carried out in this study.
The following safety measurements were performed at the pre-screening.
Medical history was assessed by an interview by the (assistant) medical investigator on the basis of the filled-in health and lifestyle questionnaire. The following clinical laboratory test were performed in fasting samples

- Haematology (RBC, WBC, differential white blood cell count, platelets, Ht, Hb, Retics)
- Serum Clinical Chemical profile: γ-GT, ALAT, ASAT, ALP, albumin, total bilirubin, urea, creatinine, glucose.
- Dipstick urinalysis (protein, glucose, leucocytes, erythrocytes, nitrite, pH, ketones, bilirubin, urobilinogen), and a microscopic inspection of sediment of urine was done.

Furthermore, a physical examination, next to blood pressure, heart rate, body weight and height measurements was conducted. Special emphasis was given to the skin of the testing areas on scars, cuts, wounds, naevi or other abnormalities.

6.7.2 Appropriateness of measurements
Appropriateness of measurements was not applicable.

6.7.3 Primary efficacy variable(s)
No efficacy variables were established in this study.

6.7.4 Topical formulation concentration measurements
Apart from the concentration measurements provided by the sponsor (Appendix 14.1.2) no other concentration measurement of the topical formulation has been made.

6.8 Data quality assurance

The Quality Assurance Unit (QAU) of TNO Quality of Life conducted audits during the study and reviewed the protocol, amendment, the final report and study documents as required by the ICH Guidelines for Good Clinical Practice (GCP). The QAU promulgated an audit certificate specifying the dates of audits and reports to management and to the principal investigator. See appendix 14.1.6.

The skin biopsy and urinary analysis were performed by the Business unit Analytical Sciences and Analytical Services, respectively in compliance with the internationally accepted standards of Good Laboratory Practice. Both units have been inspected by the Public Health Inspectorate for compliance with GLP. A written statement is supplied in the respective analytical reports (see Appendix 14.1.7.1 and 14.1.7.2).

6.9 Chemical analysis methods

All chemical analysis methods used in the study were carried using validated methods and according to the principles of GLP [4]. The analysis were carried out by, and under the responsibility of the TNO Business unit Analytical Sciences (Dr. L.P. Brüll). Detailed description of the various methods is given in Appendices 14.1.7.1 and 14.1.7.2. The following paragraphs gives a brief outline of the used methods.

6.10 Pre-preparation of skin biopsies

The collected and deeply frozen combined skin biopsy samples were firstly cut in small pieces constantly kept at cold conditions using a scalpel. Then these small pieces were transferred quantitatively to the pre-cooled dismembrator chamber, the pre-cooled grinding ball was added and the chamber was closed (B. Braun Biotech International, Melsungen, Germany). Dismembration was carried out for 5-10 seconds whereafter the chamber was opened and the resulted ‘powder’ was as quantitative as possible transferred to coded, pre-weighed and pre-cooled Eppendorf vials, closed, weighed and recorded on forms. Thereafter, these dismembrated samples were stored refigerated at -70°C and finally transferred on dry ice to the chemical analysis laboratory for analysis (see § 6.11). For the preparation of QC samples, skin derived from various donors (reconstructive surgery) was used. Various skin samples were punched out, then cut in small pieces and then further treated as decribed above. To about 45 mg of dismembrated skin, internal standard and known amounts of either Arbutin or hydroquinone was added. These QC samples were then distributed over the analysis series and analysed similarly as the unknown samples.

6.11 Chemical analysis of skin biopsy samples

The following is a brief outline of the method:
The dismembrated skin samples in Eppendorf vials were kept on dry ice and 25 µl of internal standard solution was added to the samples. Then 1 ml of acetonitril was added and the sample was mixed thoroughly vortexed for approximately 30 sec. The solution was transferred to an eppendorf cup and centrifuged at 14.000 rpm for 10 min. Sample clean up was carried out using pre-conditioned SPE columns with 3 ml acetonitril, supernatant was transferred to SPE column, effluent was collected directly, SPE was washed with 2 ml acetonitril and effluent was collected again. Thereafter, the combined

effluent was concentrated under a mild stream of nitrogen to approximately 1 ml and transferred to a derivatization vial, concentrated furthermore in the derivatization vial under a mild stream of nitrogen to approximately 500 µl, whereafter 25 µl of MSTFA reagent was added and derivatization was performed at 60° Celsius for one hour. Finally, 1 µl of the derivatization solution was injected on the high-resolution GC-MS for the determination of hydroquinone and Arbutin content. Detailed description of the analysis method and analytical conditions is given in Appendix 14.1.7.1.

6.12 Chemical analysis of urinary total hydroquinone

The following is a brief outline of the method:
Urine study samples were all thawed at room temperature, exactly 0.5 ml of urine was transferred per sample to a glass tube with screw cap, 1.5 ml ascorbic acid in water (1 mg/ml) was added, 2 ml of 10 mM citric acid/di-sodium-phosphate buffer pH 5 with 0.5 mg/ml ascorbic acid was added and finally 570 µl hydrochloride acid (37 %). The tubes were closed (not completely) with a cap and placed in a water bath at 90° C for 1 hour to break conjugation bonds.Then the tubes were cooled to room temperature and 2.2 ml 3 M TRIS buffer was added. The content of the tubes was mixed carefully and exactly 100 µl was transferred to an injection vial. Then 900 µl water was added to this vial, mixed carefully and the vial was transferred to the auto-injector of the LC-ECD system for analysis of urinary total hydroquinone content. All urinary total hydroquinone results were corrected for diuresis by their respective creatinine content. Detailed description of the LC-ECD conditions and of the analysis method is given in Appendix 14.1.7.2.

6.13 Chemical analysis of urinary creatinine

In all urine (in-study) samples the creatinine content of urine was analyzed according to a standardised enzymatic method (Jaffé method) (SOP: DAS/KLC/114) by the Clinical Chemistry working group of the Business unit Toxicology Applied Pharmacology of TNO Quality of Life.

6.14 Statistical methods planned in the protocol and determination of sample size

6.14.1 Statistical and analytical plans

Comparisons between individual treatment means of the study parameters (Arbutin and hydroquinone content of biopsies) were evaluated using a 2-sided paired Student t-test at a probability level ($p \leq 0.05$).

Detailed description of the statistical analyses are described in the statistical analysis report (SAR), see Appendix 14.8.1

In all statistical tests performed, the null hypothesis (no treatment effect) was rejected at the 0.05 level (two-sided) of probability.

Anthropometric and demographic data of the subjects are presented descriptively and tabulated.

6.14.2 Determination of sample size

The motivation of the number of 18 subjects for the repeated topical application was based on experience and on the minimal number of subjects needed to calculate reliable arithmetic means and standard deviations including getting insight in the interindividual

variation. The strategy of urine sampling and the number of samples was selected to provide sufficient information on individual levels of hydroquinone prior to application on Day 01-Day 04 following a repeated topical application and prior to biopsy on Day 01 and Day 05.

6.15 Changes in the conduct of the study or planned analyses

No changes in the conduct of the study has been made. The following changes in the planned chemical analysis has been made.
Since the analytical method for plasma Arbutin and hydroquinone did not met the set validation criteria, it was decided in mutual agreement with the sponsor not to use this method and consequently not to collect blood samples in the study. This was described in Amendment 01 and 02.

7 Study subjects

7.1 Disposition of subjects

Twenty one (21) healthy females/males participated in the study. Since all subjects arrived on time on their scheduled treatment on Day 01, it was not necessary to include any of the three assigned substitutes in the study. Finally, eighteen (18) subjects actually entered the clinical part of the study. The first subject (#01) included (FSI), entered the study on Day 01, dated 10 January 2005. The last subject (#12) out of the study (LSO) left TNO Quality of Life on Day 05, dated 14 January 2005.

7.2 Protocol deviations

The study was conducted following the mutually agreed and approved study protocol P6035 [1]. Deviations from this protocol are mentioned hereafter.

- The control and test areas were disinfected by a solution of alcohol (70% v/v) instead of 0.5% chlorhexidine digluconate in 70% ethanol.
- The skin stripped site (10 x 2 cm) for (control) biosy collection (Day 01) was sprayed with a local anaesthetic spray (Cool spray®) instead of infiltrated by a local anaesthetics consisting of 0.5% xylocaine and epinephrine (1:100000).
- On Day 05 the skin stripped site for treated biopsy collection was sprayed within a small area (3 x 2cm) using a rubber cone, with a local anaesthetic spray (Chloorethyl®) instead of infiltrated by a local anaesthetics consisting of 0.5% xylocaine and epinephrine (1:100000).
- Instead of monitoring the healing process and subjects' well-being after the biopsy procedure by a telephonic consult with the medical investigator on the seventh day following the last biopsy, subjects were all invited to visit TNO and were visually inspected by the medical investigator or the assistant medical investigator. Some subjects were invited to visit TNO for another visual inspection, others were called by the medical investigator to monitor the healing process and well-being. The results were recorded.
- The following extra calculations were made and added to the report: the total area of 3 skin biopsies (cm^2) and amount of HQ/area (table 3) and the percentage of HQ in skin (HQ/Arb+HQ) table 6.
- The urinary total hydroquinone series was re-analysed since back-calculated QC samples were deviating too much from the actual concentration. On re-analysis extra QC0 samples at 2.5 mg/l were analysed in triplicate. On re-analysis an extra CAL sample was added to the series at 1 mg/l. The re-analysis of the urine samples was cut into two batches; two series of 45 samples were analysed. Only the re-analysed series is reported in this report.

7.3 Blind breakage

Breaking the code was not applicable since this was an open study.

8 Results

8.1 Demographic and other baseline characteristics

Table 1. Demographic and other baseline characteristics of subjects (n=18) at inclusion

Gender Female (n=9)	**Mean ± SD**
Age (years)	32 ± 8
Body weight (kg)	67.6 ± 12.3
Height (m)	1.70 ± 0.08
BMI (kg/m^2)*	23.3 ± 2.6
Gender Male (n=9)	
Age (years)	27 ± 8
Body weight (kg)	77.0 ± 10.7
Height (m)	1.83 ± 0.08
BMI (kg/m^2)*	23.0 ± 2.7

8.2 Measurement of treatment compliance

On all scheduled visits at TNO (Day 01-04) topical application was carried out by nurses and were registered on study specific forms.

8.3 Efficacy results

Efficacy has been not been an objective of this study.

* BMI= Body Mass Index: is the ratio between the body weight (kg) and the square of the height in meters of a person and is a (healthy) weight index.

9 Kinetic results

Apart from protocol deviations mentioned in paragraph 7.2, this study has been conducted exactly as described in Protocol P6035 and Amendment 01, 02 and 03. In the following paragraphs the main findings are summarised. The detailed data are provided in appendices 14.1.7.1 – 14.1.7.3 as well as 14.2.1 – 14.2.5.

9.1 Topical dosing

Topical dosing has been carried out with calibrated positive displacement pipettes set at 72 µl. To apply the set amount of CP-SEN gel, topical dosing in each subject has been done by pipetting 2 times 72 µl (144 µl) of CP-SEN gel per treatment day per subject. This resulted per subject in application of, on average, 140.1 mg ± 0.1 of CP-SEN gel.

9.2 Skin biopsy Arbutin and hydroquinone results

Two series of skin samples were analysed. Samples collected on Day 01 and Day 05. Skin biopsies were immediately frozen and dismembrated as soon as possible after the biopsies were taken. Within each series a calibration line (CAL) and quality control (QC) samples for hydroquinone and Arbutin separately were freshly prepared and analysed. QC samples for hydroquinone were prepared at 2 ng and 8 ng absolute per total skin biopsy in triplicate. CAL samples included a control with internal standard and a control without internal standard and six CAL samples at 1, 2, 4, 6, 8, and 10 ng of hydroquinone absolute per total skin biopsy.
QC samples for Arbutin were prepared at 20 ng and 80 ng absolute per total skin biopsy in triplicate. Six CAL samples were prepared at 10, 20, 40, 60, 80, and 100 ng of arbutin absolute per total skin biopsy. For detailed information regarding QC samples and CAL sample results see Appendix 14.1.7.1.

9.2.1 Hydroquinone skin biopsy results

The results obtained for hydroquinone in the subjects biopsies at Day 01 were all below the lowest calibration point, this indicates that all hydroquinone levels on Day 01 were below 1.1 ng absolute per total subject skin biopsy. Since the weight of the skin biopsies differs per subject, a mean weight for biopsies has been calculated, which is 45 mg. Using this mean weight, the content of hydroquinone at Day 01 in the skin biopsies was for all subjects below the lowest CAL, i.e.22 ng skin (=LOD).
On Day 05 elevated levels of hydroquinone were determined for all subjects, ranging from 32.0 to 602 ng/g skin. It was observed that the internal standard (chlorohydroquinone) used in the analysis decreased gradually in time during the relatively long analysis run (45 hours). Hereto, especially the hydroquinone results were affected. Therefore, the average slope of this gradual decrease was established and all hydroquinone results were corrected for this phenomenon and expressed as ‘corrected analysed amount’ in the respective heading of table 2 and 3. See appendix 14.1.7.1 for more details.

The following two tables summarise the corrected results.

Table 2. Hydroquinone results of skin biopsies on Day 01

Subject Number	Weight (net) 3 skin biopsies (g)	Weight Dismembrated skin biopsies (g)	Corrected analysed amount (ng)	Corrected amount (ng/g)	Result**
01	0.0899	0.0371	-0.46	-12.4	< CAL1
02	0.0430	0.0290	-0.39	-13.4	< CAL1
03	0.0433	0.0403	-0.21	-5.3	< CAL1
04	0.0337	0.0276	-0.36	-13.2	< CAL1
05	0.0683	0.0466	-0.46	-9.9	< CAL1
06	0.0674	0.0448	-0.14	-3.2	< CAL1
07	0.0454	0.0386	-0.04	-0.9	< CAL1
08	0.0474	0.0446	-0.09	-1.9	< CAL1
09	0.0552	0.0339	-0.20	-6.0	< CAL1
10	0.0563	0.0437	-0.09	-2.1	< CAL1
11	0.0556	0.0441	0.04	0.8	< CAL1
12	0.0712	0.0513	-0.03	-0.6	< CAL1
13	**0.0501***	0.0584	0.16	2.7	< CAL1
14	0.0529	0.0489	0.06	1.2	< CAL1
15	0.0626	0.0501	0.02	0.4	< CAL1
16	0.0553	0.0539	0.01	0.1	< CAL1
17	**0.0638***	0.0639	0.09	1.4	< CAL1
18	0.0639	0.0552	0.08	1.4	< CAL1

* Dismembrated weight differs from skin biopsy weight, possibly due to adherence of water.
** CAL 1 = 1.1 ng (total skin biopsy)

Table 3. Hydroquinone results of skin biopsies on Day 05

Subject number	Weight (net) 3 skin biopsies (g)	Weight Dismembrated skin biopsies (g)	Corrected analysed amount (ng)	Corrected amount (ng/g)	Result*	Total area 3 skin biopsies (cm^2)	Amount of HQ/area (µg/cm^2)
01	0.0451	0.0423	3.56	84		0.3768	0.009
02	0.0910	0.0581	5.19	89		0.3768	0.014
03	0.0706	0.0508	9.97	196		0.3768	0.026
04	0.0585	0.0339	1.08	32		0.3768	0.003
05	0.0757	0.0556	7.41	133		0.3768	0.020
06	0.0508	0.0466	3.43	74		0.3768	0.009
07	0.0729	0.0454	4.09	90		0.3768	0.011
08	0.0425	0.0383	5.35	140		0.3768	0.014
09	0.0520	0.0341	4.56	134		0.3768	0.012
10	0.0189	0.0155	7.33	473		0.3768	0.019
11	0.0651	0.0536	1.88	35		0.3768	0.005
12	0.0444	0.0438	6.22	142		0.3768	0.017
13	0.0740	0.0452	27.21	602	> CAL6	0.3768	0.072
14	0.0335	0.0203	3.40	168		0.3768	0.009
15	0.0566	0.0467	7.56	162		0.3768	0.020
16	0.0635	0.0464	11.75	253	> CAL6	0.3768	0.031
17	0.0486	0.0400	11.42	286	> CAL6	0.3768	0.030
18	0.0683	0.0460	4.04	88		0.3768	0.011
Mean±s.d.			7.0 ± 5.9	177 ± 149			0.018 ± 0.016

* CAL 6 = 10.78 ng (total skin biopt)

All (control) skin biopsies collected on Day 01 resulted in hydroquinone levels all below CAL1, which is 1.0 ng per total skin biopt. Since no standardised amounts of dismembrated skin were analysed, all results have also been expressed relative to the weight of the dismembrated skin (ng/g) used for analysis.

From table 3 it is obvious that all skin biopsies derived from the treated site and collected on Day 05 showed elevated hydroquinone results. Three results of subjects 13, 16 and 17 were calculated by extrapolation of the calibration curve since the absolute amount of hydroquinone per skin biopsy was above the highest calibration point (CAL 6). Also here all results have been expressed relative to the weight of the dismembrated skin (ng/g). The lowest level was obtained in subject 04 (32.0 ng/g) while the highest level was found in subject 13 (602 ng/g).

9.2.2 Arbutin skin biopsy results

The results obtained for all subjects (18) on Day 01 for the Arbutin content were all below the lowest calibration point. This indicates that all Arbutin levels on Day 01 were below 8.9 ng. One subject, from whom a relative low amount of dismembrated skin was recovered, had a relatively high response for Arbutin of 200 ng/g when the total amount of Arbutin was taken relative to the weight of the dismembrated skin.

On Day 05 elevated levels of Arbutin were determined for all subjects, ranging from 863 to 9809 ng/g skin when taken relative to the weight of the dismembrated skin. The following two tables summarise the results.

Table 4. Arbutin results of skin biopsy on Day 01

Subject Number	Weight (net) 3 skin biopsies (g)	Weight Dismembrated (g)	Analysed amount (ng)	Corrected amount (ng/g)	Result**
01	0.0899	0.0371	-9.3	-249.8	< CAL1
02	0.0430	0.0290	-9.2	-317.7	< CAL1
03	0.0433	0.0403	-9.1	-224.6	< CAL1
04	0.0337	0.0276	0.0	0.0	< CAL1
05	0.0683	0.0466	-8.6	-185.2	< CAL1
06	0.0674	0.0448	-9.2	-205.3	< CAL1
07	0.0454	0.0386	-2.7	-71.1	< CAL1
08	0.0474	0.0446	-7.0	-156.2	< CAL1
09	0.0552	0.0339	**6.8**	**200.2**	< CAL1
10	0.0563	0.0437	-8.2	-186.5	< CAL1
11	0.0556	0.0441	-9.0	-203.5	< CAL1
12	0.0712	0.0513	-8.9	-174.2	< CAL1
13	**0.0501***	**0.0584**	-9.0	-153.7	< CAL1
14	0.0529	0.0489	-9.1	-186.3	< CAL1
15	0.0626	0.0501	-8.6	-171.9	< CAL1
16	0.0553	0.0539	-9.3	-172.4	< CAL1
17	**0.0638***	**0.0639**	-8.7	-136.8	< CAL1
18	0.0639	0.0552	-9.1	-165.1	< CAL1

* Dismembrated weight differs from skin biopsy weight, possibly due to adherence of water.
**CAL 1 = 8.9 ng (total skin biopt)

Table 5. Arbutin results of skin biopsy on Day 05

Subject number	Weight (net) 3 skin biopsies (g)	Weight Dismembrated (g)	Analysed amount (ng)	Corrected amount (ng/g)	Result*
01	0.0451	0.0423	129.0	**3048.7**	> CAL 6
02	0.0910	0.0581	185.1	**3185.4**	> CAL 6
03	0.0706	0.0508	209.9	**4132.8**	> CAL 6
04	0.0585	0.0339	29.3	863.0	
05	0.0757	0.0556	430.6	**7744.6**	> CAL 6
06	0.0508	0.0466	77.0	1652.6	
07	0.0729	0.0454	150.8	**3321.2**	> CAL 6
08	0.0425	0.0383	157.1	**4102.5**	> CAL 6
09	0.0520	0.0341	141.2	**4140.6**	> CAL 6
10	0.0189	0.0155	54.9	**3543.6**	> CAL 6
11	0.0651	0.0536	99.3	1851.9	
12	0.0444	0.0438	185.7	**4239.0**	> CAL 6
13	0.0740	0.0452	219.9	**4865.1**	> CAL 6
14	0.0335	0.0203	48.0	**2366.3**	
15	0.0566	0.0467	162.8	**3486.5**	> CAL 6
16	0.0635	0.0464	132.7	**2860.5**	> CAL 6
17	0.0486	0.0400	392.4	**9809.0**	> CAL 6
18	0.0683	0.0460	93.4	2031.0	

* CAL 6 = 99.46 ng (total skin biopt)

On Day 05 the absolute amount per skin biopsy for many samples was higher than the highest calibration point (CAL 6 at 99.46 ng). For subject 14 the absolute value was lower than CAL6. However, the result was high when the result was taken relative to the weight of the dismembrated skin, possibly due to the relatively low weight (20.3 mg) of skin which was recovered after dismembration. The lowest level was obtained in subject 04 (863 ng/g) while subject 17 revealed the highest result (9809 ng/g).

When the derived corrected hydroquinone amounts (ng/g) from Table 3, Day 05 were taken relative, on a weight to weight basis, to the derived corrected Arbutin+hydroquinone amounts (ng/g) results, on average a percentage of hydroquinone present in skin biopsies could be calculated. Table 6 summarises the results.

Table 6. Percentage of hydroquinone, on a weight to weight basis, present in skin

Subject number	Corrected amount of Arbutin (ng/g skin)	Corrected amount of HQ (ng/g skin)	Percentage HQ in skin (HQ/(Arb+HQ) (%)*
01	3048.7	84	2.69
02	3185.4	89	2.73
03	4132.8	196	4.53
04	863.0	32	3.57
05	7744.6	133	1.69
06	1652.6	74	4.26
07	3321.2	90	2.64
08	4102.5	140	3.29
09	4140.6	134	3.13
10	3543.6	473	11.77
11	1851.9	35	1.86
12	4239.0	142	3.24
13	4865.1	602	11.01
14	2366.3	168	6.61
15	3486.5	162	4.43
16	2860.5	253	8.14
17	9809.0	286	2.83
18	2031.0	88	4.15
Mean	**3735.8**	**177**	**4.6**
S.D.	**2137.5**	**149**	**2.9**

*** Based on a weight to weight basis**

The highest percentage of hydroquinone present in skin was established in subject 10 (11.77%) while the lowest (1.69%) was observed in subject 05. On average, a relatively small percentage of hydroquinone was present in skin (4.6% ± 2.9).

9.3 Urinary total hydroquinone results

It is well known that food contains levels of hydroquinone. Consequently, also levels of hydroquinone are present in the body. In this study subjects were not restricted into their daily habitual food and drink intake. They only were instructed to record their daily food intake using a diary (See Appendix 14.2.5, in Dutch). To record changes in urinary hydroquinone levels due to repeated topical treatment, spot urine samples were collected on a daily basis.
Prior to each application (Day 01- Day 04) or biopsy (Day 05) each subject produced a spot urine sample. In total 90 spot urine samples have been collected for urinary total hydroquinone measurements in the study. The first analysis run revealed unacceptable QC sample results. Therefore, it was decided to re-analyse the samples divided over two runs. In 24 out of 90 samples concentration of hydroquinone above the lowest calibration point (CAL 1) could be established and the QC samples met the criteria. Table 7 summarises the re-analysis results.

Tabel 7. Urinary total hydroquinone analysis results (mg/L)

Subject number	Day 01 mg/L	Day 02 mg/L	Day 03 mg/L	Day 04 mg/l	Day 05 mg/L
01	<CAL1 *	<CAL1	<CAL1	<CAL1	<CAL1
02	<CAL1	<CAL1	1.32	<CAL1	2.37
03	<CAL1	<CAL1	<CAL1	<CAL1	<CAL1
04	1.59	1.54	<CAL1	<CAL1	1.16

05	1.51	1.19	2.54	<CAL1	<CAL1
06	<CAL1	<CAL1	1.36	<CAL1	<CAL1
07	<CAL1	<CAL1	<CAL1	<CAL1	<CAL1
08	<CAL1	<CAL1	<CAL1	<CAL1	<CAL1
09	<CAL1	<CAL1	<CAL1	1.46	<CAL1
10	2.32	<CAL1	3.05	<CAL1	<CAL1
11	<CAL1	<CAL1	<CAL1	1.22	<CAL1
12	1.15	2.38	2.97	<CAL1	<CAL1
13	1.77	2.01	1.32	<CAL1	<CAL1
14	<CAL1	<CAL1	<CAL1	<CAL1	<CAL1
15	1.09	1.32	<CAL1	<CAL1	<CAL1
16	<CAL1	1.60	1.39	<CAL1	<CAL1
17	<CAL1	<CAL1	<CAL1	<CAL1	<CAL1
18	<CAL1	<CAL1	1.05	<CAL1	<CAL1

* CAL1 = 0.974 mg/L

It was observed that on Day 01, 6/18 samples, Day 02, 6/18 samples, Day 03, 8/18 samples and on Day 04 and Day 05, 2/18 samples revealed total hydroquinone levels above the lowest calibration point. Since in this study spot urine samples were collected all hydroquinone results expressed as mg/L were corrected for diuresis by the respective creatinine content of the sample. Table 8 shows the results of this correction.

Table 8. Urinary total hydroquinone results per study day per subject and corrected by creatinine for diuresis

Day 01	Hydroquinone		Creatinine	(HQ/Creat)	**Day 02**	Hydroquinone		Creatinine	(HQ/Creat)
sample code	mg/L	µmol/L	mmol/L	Ratio mmol/mol	sample code	mg/L	µmol/L	mmol/L	Ratio mmol/mol
01	<0.97	<8.85	10.35	<0.85	01	<0.97	<8.85	17.83	<0.50
02	<0.97	<8.85	15.01	<0.59	02	<0.97	<8.85	13.61	<0.65
03	<0.97	<8.85	9.85	<0.90	03	<0.97	<8.85	16.28	<0.54
04	1.59	14.44	1.71	8.45	04	1.54	13.94	1.52	9.16
05	1.51	13.67	15.05	0.91	05	1.19	10.83	12.19	0.89
06	<0.97	<8.85	11.55	<0.77	06	<0.97	<8.85	16.05	<0.55
07	<0.97	<8.85	8.34	<1.06	07	<0.97	<8.85	8.02	<1.10
08	<0.97	<8.85	15.26	<0.58	08	<0.97	<8.85	15.11	<0.59
09	<0.97	<8.85	6.14	<1.44	09	<0.97	<8.85	27.02	<0.33
10	2.32	21.08	29.56	0.71	10	<0.97	<8.85	18.62	<0.48
11	<0.97	<8.85	8.84	<1.00	11	<0.97	<8.85	6.11	<1.45
12	1.15	10.45	18.03	0.58	12	2.38	21.61	20.52	1.05
13	1.77	16.11	11.41	1.41	13	2.01	18.25	20.63	0.88
14	<0.97	<8.85	3.82	<2.32	14	<0.97	<8.85	8.15	<1.09
15	1.09	9.92	2.51	3.95	15	1.32	12.01	1.88	6.38
16	<0.97	<8.85	9.21	<0.96	16	1.60	14.52	25.43	0.57
17	<0.97	<8.85	20.11	0.44	17	<0.97	<8.85	16.88	<0.52
18	<0.97	<8.85	6.75	<1.31	18	<0.97	<8.85	7.70	<1.15

Table 8. Continue

Day 03 sample code	Hydroquinone		Creatinine	(HQ/Creat)	**Day 04 sample code**	Hydroquinone		Creatinine	(HQ/Creat)
	mg/L	µmol/L	mmol/L	Ratio mmol/mol		Mg/L	µmol/L	mmol/L	Ratio mmol/mol
01	<0.97	<8.85	10.04	<0.88	01	<0.974	<8.85	6.04	<1.46
02	1.32	11.96	11.95	1.00	02	<0.974	<8.85	20.70	<0.43
03	<0.97	<8.85	18.04	<0.49	03	<0.974	<8.85	19.96	<0.44
04	<0.97	<8.85	5.79	<1.53	04	<0.974	<8.85	4.99	<1.77
05	2.54	23.06	17.26	1.34	05	<0.974	<8.85	14.49	<0.61
06	1.36	12.36	5.80	2.13	06	<0.974	<8.85	18.07	<0.49
07	<0.97	<8.85	2.11	<4.19	07	<0.974	<8.85	10.85	<0.82
08	<0.97	<8.85	17.52	<0.50	08	<0.974	<8.85	22.39	<0.40
09	<0.97	<8.85	9.06	<0.98	09	1.458	13.24	24.45	0.54
10	3.05	27.72	17.37	1.60	10	<0.974	<8.85	7.49	<1.18
11	<0.97	<8.85	10.77	<0.82	11	1.224	11.11	19.61	0.57
12	2.97	27.01	20.49	1.32	12	<0.974	<8.85	1.66	<5.53
13	1.32	12.01	11.85	1.01	13	<0.974	<8.85	5.80	<1.53
14	<0.97	<8.85	11.35	<0.78	14	<0.974	<8.85	6.87	<1.29
15	<0.97	<8.85	9.82	<0.90	15	<0.974	<8.85	8.30	<1.07
16	1.39	12.64	21.77	0.58	16	<0.974	<8.85	7.66	<1.15
17	<0.97	<8.85	7.28	<1.22	17	<0.974	<8.85	15.77	<0.56
18	1.05	9.55	12.57	0.76	18	<0.974	<8.85	5.70	<1.55

Table 8. Continue

Day 05 sample code	Hydroquinone		Creatinine	(HQ/Creat)
	mg/L	µmol/L	mmol/L	Ratio mmol/mol
01	<0.97	<8.85	10.70	<0.83
02	2.37	21.56	17.80	1.21
03	<0.97	<8.85	16.14	<0.56
04	1.16	10.52	1.19	8.82
05	<0.97	<8.85	27.03	<0.33
06	<0.97	<8.85	14.32	<0.62
07	<0.97	<8.85	9.18	<0.96
08	<0.97	<8.85	9.82	<0.90
09	<0.97	<8.85	12.68	<0.70
10	<0.97	<8.85	8.06	<1.10
11	<0.97	<8.85	4.37	<2.02
12	<0.97	<8.85	2.31	<3.83
13	<0.97	<8.85	6.89	<1.28
14	<0.97	<8.85	5.66	<1.56
15	<0.97	<8.85	3.85	<2.30
16	<0.97	<8.85	12.29	<0.72
17	<0.97	<8.85	6.74	<1.31
18	<0.97	<8.85	10.04	<0.88

The highest hydroquinone/creatinine ratio (9.16 mmol/mol) was observed on Day 02 (subject 04). Subject 04 showed also the highest levels on Day 01 and Day 05.

A large variation in urinary total hydroquinone levels corrected for creatinine was observed in the collected spot urine samples. Based thereon, changes in urinary total HQ levels due to topical treatment of Arbutin could not be established.

9.4 Statistical analysis results

The statistical analyses were performed according to the statistical plan (§ 6.14.1) on differences between the skin data results of Day 01 and Day 05 for arbutin and hydroquinone. All analyses were performed using SAS V8 software package.
The differences between Day 01 and Day 05 were analysed using ANOVA, according to the following ANOVA table:

Source	*Df*
Subject	18-1
Moment	2-1
Residual	17
Total	35

The ANOVA test shows significant differences between Day 01 and Day 05 for all variables, except for Arbutin : weight dismembrated and Hydroquinone: weight dismembrated (note: these two variables were derived from identical samples).
The inspection of the residual plots revealed, however, severe violations of the assumptions for ANOVA. Therefore, it was deceided to perform a nonparametric Signed Rank test as well.
The Signed Rank test assumes that the distribution is symmetric (skewness ~ 0). The signed rank statistic is computed as $S = \Sigma r_i^+ - n_t(n_t + 1)/4$ where r^+_i is the rank of $|y_i - \mu_0|$ after discarding y_i values equal to μ_0, and the sum is calculated for values of $y_i > \mu_0$. Average ranks are used for tied values. The *p*-value is the probability of obtaining a signed rank statistic greater in absolute value than the absolute value of the observed statistic *S*. As $n_t <= 20$, the *p*-value of the statistic *S* is computed from the exact distribution of *S* (definition of test obtained from SAS online doc).
The difference between Day 05 and Day 01 were calculated and used for the analyses. Because the logaritmic values of the differences between Day 01 and Day 05 had skewness values closer to zero (which is an assumption in order to perform a Signed Rank test), this was also analysed. The results of the non-parametric Signed Rank are presented in table 9.

Table 9. Statistical (Signed Rank test) results of Arbutin and hydroquinone (HQ) in skin biopsies (Day 01 vs Day 05)

VarName	Skewness	P-value (RankSign test)
Arbutin and HQ: Weight Dismembrated [g]		
Diff	-0.09	0.5872
Difflog	-0.14	0.5509
Arbutin and HQ: Analysed amount [ng]		
Diff	1.43	<.0001
Difflog	-0.47	<.0001
Arbutin and HQ: Corrected amount [ng/g]		
Diff	1.62	<.0001
Difflog	-0.46	<.0001
Arbutin and HQ: Official result [ng/g]		
Diff	2.06	<.0001
Difflog	-1.16	<.0001

The statistical test shows significant differences between Day 01 and Day 05 for all variables ($p < .0001$), except for the variable: weight dismembrated (p=0.5872) for Arbutin and Hydroquinone since this result was obtained from the same sample. See Appendix 14.1.8 for the statistical analysis report (SAR).

10 Safety results

10.1 Extent of Exposure

In the present study 18 subjects were topically treated with CP-SEN gel containing 6.3% (w/w) Arbutin. Hereto, two times a volume of 72 µL of the formulation (=140.1 mg) was applied by a calibrated positive displacement pipet in small evenly-spaced blobs within a delineated area of 50 cm^2. Thereafter the product was spread-out evenly by hand (index finger) covered with a disposable glove using circular motions until the product has blended with the skin.

10.2 Adverse Events

AEs were established by the medical investigator on basis of:
1. Answer to the open question: 'how are you feeling?'
2. Spontaneous reporting
3. Well-being questionnaire (Form P6035 F05; Appendix 14.1.1)

AEs were classified by the medical investigator according to ICD-10, published by the WHO Collaborating Centre for International Drug Monitoring. The medical investigator registered the findings, conclusions and actions according to TNO standard procedures on forms F01 and F02.

Expected study design related side effects
Due to the biopsy taking little skin wounds resulted and a slight pain and discomfort in this skin area occurred. These expected study design related side effects were therefore not denoted as an adverse event.

In all subjects redness of the left buttock at the site of local anesthesics was observed. The date at which this redness was at first observed visually is taken as start date of the adverse event. All subjects were instructed by the dermatologist on Day 01 to leave the hemostatic sponges and the covered plaster in place for at least two days and/or to wait until the plaster dropped off spontaneously. Consequently, the starting day of this adverse event varies between subjects and it is likely that the real starting date of the redness could have been 10 January, 2005. Since all these redness adverse events were unexpected, subjects were informed verbally on Day 05 prior to skin biopsy by the principal investigator about these results. They were also informed that for Day 05 a slightly altered biopsy procedure was going to be followed (spraying a smaller area within a rubber cone and another local anaesthetics) and subjects were instructed to visit TNO 1.5 week after Day 01 for a) a visual inspection of the red spots, b) to monitor the healing process and c) to establish their well-being, rather then a telephonic consult with the medical investigator. It was decided to take the date of this extra control visit as end date of the redness when a sufficient healing tendency could be observed. Otherwise an appointment for a follow-up telephonic consult was made with subjects. Finally on 02 February 2005 the last subject reported that the experienced adverse events were recovered without a complaint left. Since in this study the daily topical application took place on the right buttock, no changes to the study (intervention) due to these adverse event had to be made.

The following table summarises the Adverse Events per subject:

Table 10. Reported AEs, ICD code, start and end dates, severity, relation to treatment and trial design, and medication taken, per subject

Entry Number	ICD code	Adverse event	Start date	End date	Severity	Relation to treatment*	Relation to trial design*	Medication taken
13	Y84.8	Redness left buttock	11-01-2005	20-01-2005	Moderate	5	1	flammazine
14	Y84.8	Redness left buttock	11-01-2005	02-02-2005	Moderate	5	1	flammazine
16, 15, 18	Y84.8	Redness left buttock	12-01-2005	20-01-2005	Moderate	5	1	flammazine
17	Y84.8	Redness left buttock	12-01-2005	27-01-2005	Moderate	5	1	flammazine
06	Y84.8	Redness left buttock	12-01-2005	24-01-2005	Mild	5	1	
11	Y84.8	Redness left buttock	12-01-2005	18-01-2005	Mild	5	1	
10	Y84.8	Redness left buttock	13-01-2005	28-01-2005	moderate	5	1	flammazine
01, 02, 03 04, 05, 08 09, 12	Y84.8	Redness left buttock	13-01-2005	20-01-2005	Mild	5	1	
07	Y84.8	Redness left buttock	14-01-2005	20-01-2005	Mild	5	1	
03	Y84.8	Bleeding biopsy spots right buttock	14-01-2005	14-01-2005	Mild	5	1	
05	Y84.8	Bruise left buttock	13-01-2005	Continued after 20-01-2005	Mild	5	1	
05	Y84.8	Bruise right buttock	13-01-2005	Continued after 20-01-2005	Mild	5	1	
09	L98.9	2 small red spots right buttock	11-01-2005	13-01-2005	Mild	4	5	
10	Y84.8	Wound left buttock due to removal of plaster	14-01-2005	19-01-2005	moderate	5	1	
11	J11.1	Influenza-like disease	13-01-2005	15-01-2005	Mild	5	5	
12	J00	Common cold	13-01-2005	Continued after day 05	Mild	5	5	
13	Y84.8	Pain surrounding biopsy spots left buttock	10-01-2005	20-01-2005	moderate	5	1	
14	R53	Slight fatique	10-01-2005	11-01-2005	Mild	4	5	
14	Y84.8	Pain surrounding biopsy spots left buttock	11-01-2005	20-01-2005	moderate	5	1	
14	Y84.8	Slight redness right buttock	20-01-2005	27-01-2005	Mild	5	1	
16	Y84.8	Bleeding biopsy spots right buttock	14-01-2005	14-01-2005	Mild	5	1	
16	Y84.8	Wound left buttock due to removal of plaster	13-01-2005	20-01-2005	Mild	5	1	
17	Y84.8	Pain surrounding biopsy spots left buttock	10-01-2005	12-01-2005	Mild	5	1	
17	Y84.8	Wound left buttock due to removal of plaster	14-01-2005	20-01-2005	Mild	5	1	
18	Y84.8	Bleeding biopsy spots left buttock	10-01-2005	10-01-2005	Mild	5	1	
18	Y84.8	Bleeding biopsy spots right buttock	15-01-2005	16-01-2005	Mild	5	1	
18	Y84.8	Wound left buttock due to removal of plaster	13-01-2005	20-01-2005	Mild	5	1	

*1= definitely, 2= probable, 3= possible, 4= unlikely, 5= not related, 6= not assessed

10.3 Deaths, other serious adverse events and other significant adverse events

None observed.

10.4 Clinical laboratory evaluation

As judged by the medical investigator no significant clinically relevant laboratory pre-study check up results were reported (see appendix 14.2.5).

10.5 Vital signs, physical findings and other observations related to safety

No abnormal or significant clinically relevant results were recorded in heart rate, blood pressure, and physical examinations in the pre-study check up.

10.6 Safety summary and conclusions

Repeated topical application of CP-SEN gel containing 6.3% (w/w) Arbutin was well tolerated by all subjects in this study. Investigations of vital signs, as well as clinical laboratory parameters were judged as 'normal' by the medical investigator as expected for healthy subjects.
Most AEs reported were predominantly related to the study design (skin biopsy). Especially all subjects experienced skin redness of the left buttock when Cool spray® as the local anaesthetic was used on Day 01. No such AEs were reported on Day 05 when Chloorethyl spray® as the local anaesthetic was used in combination with a smaller sprayed area. A final conclusion for the redness on Day 01 is at present unknown.

11 Discussion and conclusions

This report describes the conduct and the results of a human volunteer study which was aimed to establish skin metabolism of Arbutin to hydroquinone of this active ingredient, after a repeated topically applied formulation (CP-SEN gel). The skin metabolism was evidenced by the establishment of Arbutin and hydroquinone amounts in skin biopsies taken from a treated area (right buttock) of 50 cm^2 after repeated application (140.1 mg per treatment) for consecutive 4 days and compared to skin biopsies taken from an untreated contralateral (control) site (left buttock). The secondary study objective was to confirm changes of total hydroquinone levels in spot urine samples collected prior to biopsy (Day 01 and Day 05) and prior to application (Day 02 – Day 04).

Investigations of vital signs, as well as clinical laboratory parameters were judged as 'normal' by the medical investigator as expected for healthy subjects. In all subjects redness of the left buttock at the site of local anesthesics was observed. Finally on 02 February 2005 the last subject reported that the experienced adverse events were recovered without a complaint left. Repeated topical application of CP-SEN gel containing 6.3% (w/w) Arbutin was well tolerated by all subjects in this study.

A total of 2*18*3=108 skin biopsies were collected and 5*18=90 urine samples. In these samples either hydroquinone and Arbutin levels (biopsies) or total hydroquinone and creatinine levels (urine) were established.

It was observed that in all control biopsies low levels (<1.1 ng) of hydroquinone per skin biopsy could be established. Suggesting that the actual concentration of hydroquinone in untreated skin in man is low.
Higher levels of hydroquinone, on average 177 ± 149 ng/g (range: 32.0 – 602 ng/g) were found in the biopsies derived from the treated area when taken relative to the dismembrated weight. This indicates that repeated skin treatment with CP-SEN gel for 4 days leads to skin absorption of Arbutin followed by partly metabolism into hydroquinone.

Furthermore, it was observed that for all control skin biopsies, except for subject 09, collected on Day 01 of the study, Arbutin levels were below 8.9 ng, when the results were taken relative to the corresponding dismembrated skin weights. This leads to the conclusion that concentrations of Arbutin in skin in man is low. Only the skin biopsy sample of subject 09 revealed a positive result of 200 ng/g.
In 5 out of 18 treated samples elevated levels of Arbutin could be established, the lowest level (863 ng/g) was established in subject 04 while the highest level (9809 ng/g) was found in subject 17. On average, 3736 ng/g ± 2138 (range: 863-9809 ng/g) could be established in these samples. This suggests that repeated treatment of CP-SEN gel leads to skin absorption and to elevated levels of Arbutin in skin compared to untreated skin.

Based on the achieved Arbutin and hydroquinone skin results one can calculated, on a weight to weight basis, the percentage of the hydroquinone content of these samples by taken hydroquinone relative to the Arbutin+hydroquinone content. On average, this amounted to 4.6 % ± 2.9; range 1.69% - 11.77.

The actual levels of hydroquinone established in treated skin (µg/cm^2) amounted, on average, to 0.018 ± 0.016 (µg/cm^2), range 0.003 – 0.072 (µg/cm^2). This is a relatively small figure for which the contribution to the total body burden can be neglected.

Differences between the skin data results of Day 01 and Day 05 for arbutin and hydroquinone were statistically analysed. Because the logaritmic values of the differences between Day 01 and Day 05 had skewness values closer to zero (which is a condition in order to perform a Signed Rank test), this was also analysed. The statistical tests showed significant differences between Day 01 and Day 05 for all variables ($p < .0001$), except for the variable: weight dismembrated (p=0.5872) for Arbutin and hydroquinone since this result was derived from the same skin sample.

It is well known that food contains hydroquinone levels. Consequently, also the human body contains certain levels of hydroquinone. The latter is evidenced by urinary HQ levels. Therefore, in this study spot urine sampling has been carried out on a daily basis just prior to topical application to investigate possible changes in urinary HQ levels due to the repeated topical application. In total 90 spot urine samples have been collected for urinary hydroquinone measurements in this study. In 24 out of 90 samples a concentration of total hydroquinone above the lowest calibration point (0.974 mg/L) could be established. It was observed that on Day 01, 6/18 samples, Day 02, 6/18 samples, Day 03, 8/18 samples and on Day 04 and Day 05, 2/18 samples revealed hydroquinone levels above the lowest calibration point. The collected spot urine samples were corrected for diuresis by the respective creatinine content of the sample. The highest hydroquinone/creatinine ratio (9.16 mmol/mol) was observed on Day 02 (subject 04). Furthermore, subject 04 showed also the highest levels on Day 01 (8.45 mmol/mol), Day 02 (9.16 mmol/mol) and Day 05 (8.82 mmol/mol). Based on the large variation in the established urinary total HQ results, changes in urinary HQ levels due to topical treatment of Arbutin could not be established.

From the derived skin sample results it is obvious that in untreated skin (very) low levels of either hydroquinone or Arbutin could be detected. When the skin is treated for 4 days with an Arbutin containing gel (CP-SEN), elevated levels of hydroquinone (mean= 177 ng/g) and Arbutin (mean=3736 ng/g) were detected.

The following conclusions can be drawn from this study:

- In all control skin biopsy samples very low levels of hydroquinone (< 1.1 ng) and Arbutin (< 8.9 ng) per skin biopsy were present;
- In all treated skin samples hydroquinone, on average 177 ± 149 ng/g (range: 32.0 – 602 ng/g) and Arbutin, on average, 3736 ng/g ± 2138 (range: 863-9809 ng/g) could be established when taken relative to the dismembrated weight;
- Repeated topical application of CP-SEN gel containing 6.3% (w/w) Arbutin leads to detectable amounts of Arbutin and hydroquinone in skin;
- The statistical tests showed significant differences ($p < .0001$) between Day 01 and Day 05 for the variables: analysed amount (ng) and corrected amount (ng/g) for hydroquinone as well as for Arbutin;
- In a number of spot urine samples (24/90) detectable hydroquinone levels, corrected by creatinine for diuresis (range: < 4.19 – 9.16 mmol/mol), were established;
- Based on the large variation in the established urinary total HQ results, changes in urinary HQ levels due to topical treatment of Arbutin could not be established;
- When the hydroquinone content in the skin samples is taken relative, on a weight to weight basis, to the Arbutin+hydroquinone content, on average 4.6% (± 2.9) (range: 1.69-11.77) of hydroquinone is present in these skin samples.
- Actual levels of hydroquinone in treated skin amounted on average to 0.018 ± 0.016 $\mu g/cm^2$, range 0.003 – 0.072;
- Repeated topical application of CP-SEN gel containing 6.3% (w/w) Arbutin was well tolerated by all subjects in this study.

12 Tables and figures referred to but not included in the text

12.1 Summary tables

None

12.2 Figures

None

13 References

1. TNO Protocol P6035 ”Skin metabolism and potential absorption after repeated topical application of Arbutin in human volunteers” Revised Final 1, dated 22 November 2005.

2. Amendment 1 to Protocol P6035 ”Skin metabolism and potential absorption after repeated topical application of Arbutin in human volunteers” Final 1, dated 04 January 2005.

3. Amendment 2 to Protocol P6035 ”Skin metabolism and potential absorption after repeated topical application of Arbutin in human volunteers” Final 1, dated 07 January 2005.

4. Amendment 3 to Protocol P6035 ”Skin metabolism and potential absorption after repeated topical application of Arbutin in human volunteers” Final 1, dated 24 March 2005.

5. Organisation for Economic Co-Operation and Development OECD Principles of Good Laboratory Practice (GLP) (as revised 1997). Paris. (ENV/MC/CHEM (98)17.

14 Appendices

14.1 Study information

14.1.1 Protocol P6035 and Amendments 01, 02 and 03

14.1.2 CofA and MSDS of CP-SEN gel

14.1.3 List of METC members and letter(s) of approval

14.1.4 Sample informed consent (blank)

14.1.5 CV of Principal Investigator and Medical Investigator

14.1.6 Audit certificate

14.1.7 Bio-analytical reports (n=2)

14.1.7.1 The quantitative determination of hydroquinone and Arbutin in skin biopsies: sample analysis. Appendix 14.1.7.1 to TNO Report V6035. Final, dated 12 May 2005

14.1.7.2 The quantitative determination of hydroquinone in acid hydrolysed urine: sample analysis. Appendix 14.1.7.2 to TNO Report V6035. Final, dated 12 May 2005

14.1.8 Statistical Analysis Report (SAR)

14.2 Individual subject data listings

14.2.1 Individual skin biopsy results Arbutin and hydroquinone (spreadsheets)

14.2.2 Individual urinary creatinine results

14.2.3 Individual urinary hydroquinone results corrected for diuresis (spreadsheet)

14.2.4 Individual clinical laboratory data (pre-screening)

14.2.5 Individual Daily Food and Drink Intake questionnaires

14.3 Case Report Forms

Not applicable

28-day Repeated-dose Oral Toxicity Study of Arbutin in Rats with a 28-day Recovery Period

28-day Repeated-dose Oral Toxicity Study of Arbutin in Rats with a 28-day Recovery Period

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1. Introduction

This study evaluated the 28-day repeated-dose oral toxicity of Arbutin in rats with a recovery period.

2. Administration Period of Test Substance

Administration period: Sept. 10 to Oct. 9, 1986
Recovery period: Oct. 8 to Nov. 6, 1986

3. Materials and Methods

3.1 Animals

Male and female SPF Sprague-Dawley (SD) rats (Crj: CD, Charles River Japan Inc.) were purchased at 4 weeks of age. After a one-week acclimation period, animals appearing normal were divided into groups with equal average body weight. Body weight was 111 to 129 g in male rats and 95 to 113 g in female rats at the start of the study.

3.2 Housing conditions

Animals were housed throughout the acclimation and test periods in a barrier facility. Temperature and humidity of the animal quarters were maintained at 23 ± 2ºC and 55 ± 5% relative humidity, respectively, with an air exchange frequency of 32 times/hour and a light cycle of 12 hours. Rats were housed in suspended wire-mesh metal cages (300 x 200 x 400 mm: Clea Japan Inc., Tokyo, Japan), two each per cage. They were fed laboratory chow (radiation-sterilized, NMFR: Oriental Yeast Co., Ltd.) and tap water (ultraviolet ray and microfilter-treated) *ad libitum*.

3.3 Test substance

Arbutin (Lot a) was used as the test substance.

3.3.1 Preparation of test substance

Once per week, the required quantity of test substance was weighed out and dissolved by heating in an appropriate amount of water for injection (Hikari Pharmaceutical Co.). Aliquots of the prepared test substance were dispensed into amber screw-cap bottles and stored at room temperature.

3.4 Dosage groups and administration method

A pilot 10-day toxicity study was conducted to determine doses for this study. Solubility of the test substance in water increases with temperature, and is 25% (w/v) at 37ºC (body temperature in rats). Concentrations of test substance in vehicle were as follows in the pilot study: 25% (w/v) as maximum, 20% (w/v), 15% (w/v) and vehicle control. Dose volume was fixed at 10 ml/kg. No test substance-related abnormalities were observed in clinical signs, body weight, food consumption, serum chemistry, organ weight or histopathology in any animal in the pilot study.

In the pilot study, it was necessary to heat the 25% and 20% concentrations of the test substance. A small amount of precipitation, nevertheless, was observed in syringes containing the 25% (w/v) concentration when cooled. To assure accurate dosing, the maximum concentration was set at 20% (w/v) in the present study. The maximum practicable dose volume for a 28-day toxicity study was considered to be 5 ml/kg/day. The high dose level was therefore set at 1000 mg/kg/day. Middle and low dose levels were 200 and 40 mg/kg/day, calculated at a common ratio of 5, for a total of 4 groups, including a vehicle control group. Prepared test substance was orally administered at a dose volume of 5 ml/kg once daily 7 days per week for 4 weeks. Each group consisted of 16 males and 16 females. Six animals of each sex in each group were scheduled for a 28-day recovery after completion of dosing.

3.5 Observations

3.5.1 Clinical signs, body weight, and food consumption

Clinical signs were monitored at every dosing. Body weight and 24-hour food consumption were measured once per week.

3.5.2 Hematology

Blood for hematology was collected under ether anesthesia from the abdominal aorta. Edetate dipotassium was added to inhibit coagulation. Hematological parameters included red blood cell, white blood cell and platelet counts (by electrical impedance), hemoglobin (cyanmethemoglobin method), mean red blood cell volume (MCV), mean red blood cell hemoglobin (MCH), mean red blood cell hemoglobin concentration (MCHC), and hematocrit by an automatic hemocytometer (Model CC-180A, Toa Iyo Denshi Co., Ltd.). Reticulocyte count (new methylene blue stain) and white blood cell differential count (May-Giemsa stain) were also performed.

3.5.3 Serum chemistry

Blood collected under ether anesthesia from the abdominal aorta was allowed to clot for 30 to 40 minutes at room temperature, and centrifuged (at 3000 rpm, 15 min.). Serum was sent to Toukuri Laboratory to measure the following analytes: total protein (Biuret method), A/G ratio (Biuret method, BCG method), GOT, GPT (UV method), ALP (GSCC compliance), total cholesterol (enzymatic method), triglyceride (enzymatic method), blood urea nitrogen (urease-GLDH method), creatinine (Jaffe method), glucose (enzymatic method), Na^{+}, K^{+} and Cl^{-} (electrode method), Ca^{++} (O-CPC method), and inorganic phosphorous (enzymatic method). An autoanalyzer (Hitachi Model 736) was used for serum chemistry.

3.5.4 Urinalysis

Urinalysis was conducted during the 4th week of dosing and in the recovery periods using fresh urine collected by abdominal compression. Urinalysis test paper (Miles-Sankyo, N-multi-sticks III) was used to estimate pH, protein, glucose, ketone bodies, bilirubin, occult blood, nitrite, and urobilinogen.

Specific gravity was measured using a serum protein refractometer (Atago-sha).

3.5.5 Pathology

Animals were exsanguinated prior to necropsy.

The following organ weights were measured in all animals: brain, pituitary gland, salivary gland, thymus, heart, liver, spleen, kidney, adrenal gland, prostate, testis, and ovary. Relative organ weight was calculated by dividing by body weight on the day of necropsy. In addition to measured organs, the following tissues from the control group and high-dose group were fixed in 10% buffered formalin: skin, parotid gland, trachea, thyroid gland, tongue, esophagus, stomach, small intestine, large intestine, mesenteric lymph nodes, cervical lymph nodes, pancreas, urinary bladder, seminal vesicle, uterus, vagina, Harderian gland, eye, femur, and spinal cord. Tissues and organ specimens were imbedded in paraffin, blocked and sectioned, and stained with hematoxylin and eosin for histopathology. Pathological examination of animals in the 200 and 40 mg/kg groups and all animals in recovery groups was conducted as indicated.

3.5.6 Statistical methods

Quantitative parameters were evaluated by a Student’s t-test (normal distribution) or Welch’s modification of the Student’s t-test (skewed distribution). A rank-sum test (Mann-Whitney U test) was used for semi-quantitative urinalysis values.

4. Results

4.1 Clinical signs

All animals survived and no abnormalities were observed in any group during the observation period.

4.2 Body weight

Fig. 1 shows body weights during the observation period.

The trend of body weight in male rats was similar among all groups. The trend for female rats resembled that for male rats during the dosing period. Body weight gain was reduced in females at 1000 mg/kg in Weeks 5 and 6 (in the recovery period), but recovered during and after Week 7.

4.3 Food consumption

Food consumption is shown in Fig. 2.

No significant difference between control and dosed groups was observed during the observation period.

4.4 Hematology

Table 1 displays hematology results at the end of the dosing period.

Decreases in hematocrit and in counts for red blood cells, white blood cells and platelets, and increases in MCV and MCH, were observed in males at 200 mg/kg. No significant difference between control and dosed groups was observed in female rats.

Table 2 displays hematology results at the end of the recovery period.

A decrease in neutrophil and monocyte ratios was observed in males at 200 mg/kg. Increases in MCH, MCHC and lymphocyte ratio, and a decrease in neutrophil ratio were found in male rats at 1000 mg/kg. No significant difference between control and dosed groups was observed in female rats.

4.5 Serum chemistry

Table 3 shows serum chemistry results at the end of the dosing period.

An increase in Na^{+} was observed in male rats at 200 mg/kg. A decrease of Cl^{-} was seen in male rats at 1000 mg/kg. An increase in ALP was found in females at 40 mg/kg and an increase in total cholesterol was observed in females at 1000 mg/kg.

Table 4 displays blood biochemistry results at the end of the recovery period.

An increase in Ca^{++} was observed in males at 200 mg/kg. No significant difference between control and dosed groups was observed in female rats.

4.6 Urinalysis

Table 5 displays results for urinalysis in the last week of the dosing period.

An increase in specific gravity of urine was observed in male rates at 40 and 1000 mg/kg. A decrease in pH was seen in female rats at 40 mg/kg.

Table 6 shows results for urinalysis in the recovery period. No significant difference between control and dosed groups was observed for either sex.

4.7 Necropsy findings

Diffuse hemorrhage in the thymus of a male rat in the control group was observed at the end of the dosing period. Submucosal hemorrhage in the cecum was observed in a female at 200 mg/kg.

At the end of the recovery period, a male in the 40 mg/kg group had a diaphragmatic hernia in which a part of liver perforated through the diaphragm into the thoracic cavity.

No changes were observed in other animals.

4.8 Organ weights

Tables 7 and 8 show absolute and relative organ weights at the end of the dosing period. An increase in brain weight was observed in male rats at 40 mg/kg. An increase in absolute and relative weights of adrenal glands was observed in female rats at 40 mg/kg.

Tables 9 and 10 show absolute and relative organ weights at the end of the recovery period.

A decrease in relative weight was observed for the seminal vesicle at 40 and 200 mg/kg. A decrease in the absolute weight of brain and an increase in relative weight of salivary gland were found in female rats at 200 mg/kg. Decreased weights of adrenal gland and ovary were observed at 1000 mg/kg.

4.9 Histopathology

Because no gross changes associated with the test substance were observed at necropsy at the end of the dosing period, histopathological examination was done only in control and the high-dose groups at the time. Granuloma was sporadically observed in the livers of males and females from both groups. This finding was interpreted as spontaneous, and no other histopathological finding associated with the test substance was observed.

5. Discussion

The subacute toxicity of Arbutin was evaluated by repeated oral administration at dose levels of 40, 200, and 1000 mg/kg/day. Groups included animals allowed a 28-day recovery after dosing.

No test substance-related clinical sign or death was observed. Body weight did not differ between control and dosed groups in either sex during the dosing period. During the recovery period, a slight reduction of body weight was observed in females at 1000 mg/kg compared with the control group during Weeks 5 and 6, which was considered as an incidental change in light of the recovery during Week 7.

No significant difference between control and dosed groups was observed in food consumption during the test period.

Hematological examination at the end of the dosing period revealed decreases in hematocrit and counts of red blood cells, white blood cells and platelets, and increases in MCV and MCH were observed in males at 200 mg/kg. At the end of the recovery period, a decrease in neutrophil and monocyte ratios was also observed in this group. Increases in MCH, MCHC and lymphocyte ratio, and a decrease in neutrophil ratio were observed in males at 1000 mg/kg. These changes, however, were within normal ranges and are not considered as effects of test substance.

Serum chemistry analysis at the end of the dosing period revealed an increase in Na^{+} in males at 200 mg/kg and a decrease in Cl^{-} in males at 1000 mg/kg. An increase of ALP was observed in females at 40 mg/kg and an increase in total cholesterol was seen in females at 1000 mg/kg. An increase in Ca^{++} was observed at the end of the recovery period in males at 200 mg/kg. These results were within normal range and are not considered as being affected by the test substance.

Urinalysis during the last week of the dosing period revealed an increase in specific gravity in males at 40 mg/kg and 1000 mg/kg, and a decrease in pH in females at 40 mg/kg. Because these changes were slight and within the physiological ranges, they are considered spontaneous. There was no significant difference between control and dosed groups during the last week of the recovery period.

At necropsy at the end of the dosing period, a male in the control group had diffuse hemorrhage in the thymus, and a female in 200 mg/kg had submucosal hemorrhage in the cecum. At the end of the recovery period, a male in the 40 mg/kg group had diaphragmatic hernia in which a part of liver perforated through the diaphragm to the thoracic cavity. These findings were considered spontaneous, and did not display any relationship with test substance.

An increase in relative organ weight was observed at the end of the dosing period in the brains of males at 40 mg/kg. Increases were seen for both absolute and relative weight of adrenal glands in females at 40 mg/kg. A decrease in relative weight of seminal vesicle was observed at 40 and 200 mg/kg at the end of the recovery period. A decrease in absolute brain weight and an increase in relative weight of salivary gland were found in females at 200 mg/kg. A decrease in the absolute weights of adrenal gland and ovary was observed at 1000 mg/kg. These changes were within the normal range and are not considered to be effects of the test substance.

There were no histopathological findings associated with the test substance.

In conclusion, changes observed in this study were regarded as normal physiological variations or spontaneous findings, and there are no changes attributed to the test substance.

6. Conclusion

Repeated-dose oral toxicity of Arbutin was evaluated in rats during a 28-day administration period and with a recovery period. No changes attributed to Arbutin were observed in any parameter in any group. The “no observed effect” level of Arbutin is estimated to be at least 1000 mg/kg/day.

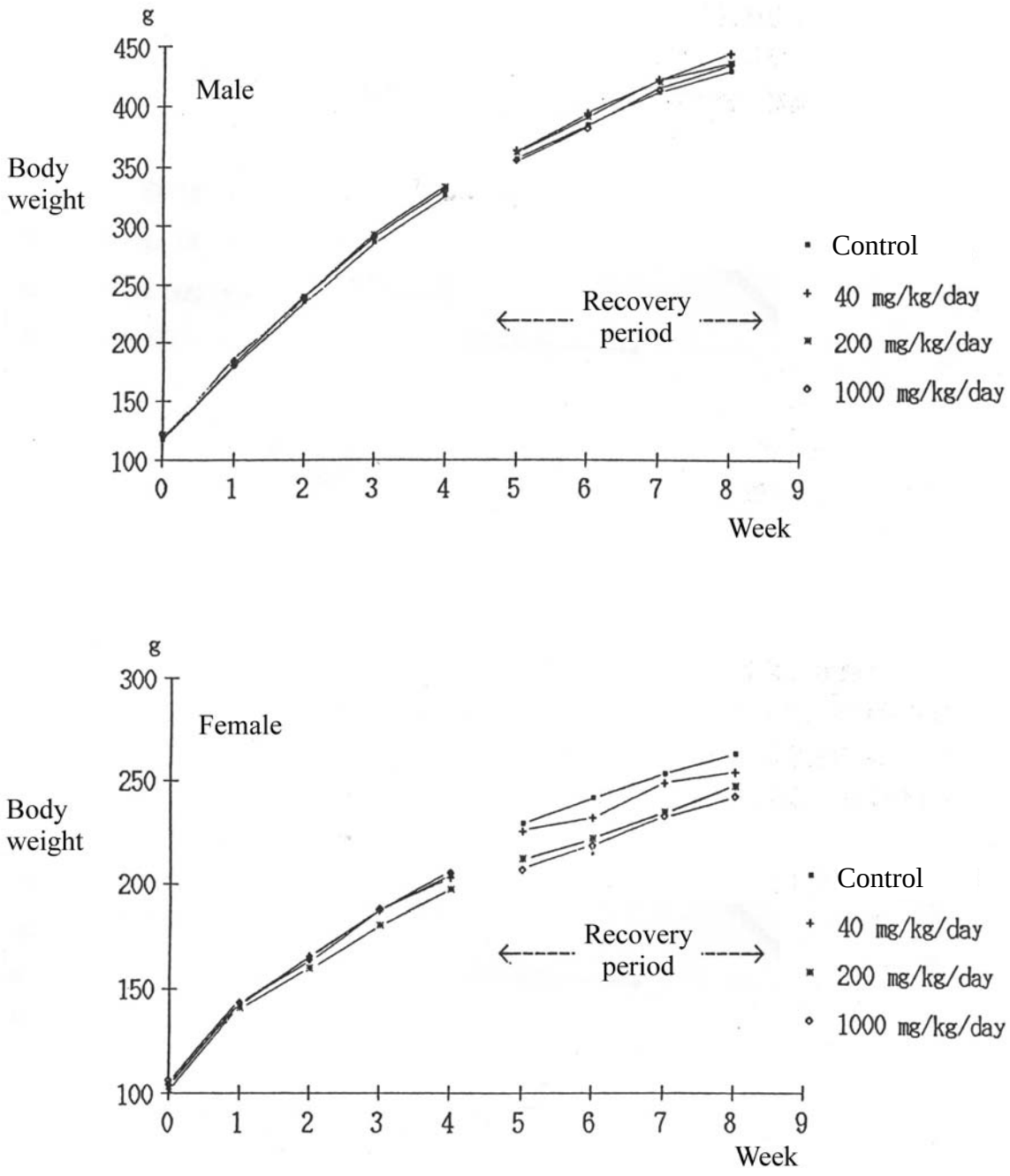

Fig. 1 Body weight of SD rats during 28 days of daily oral administration of Arbutin and subsequent recovery period

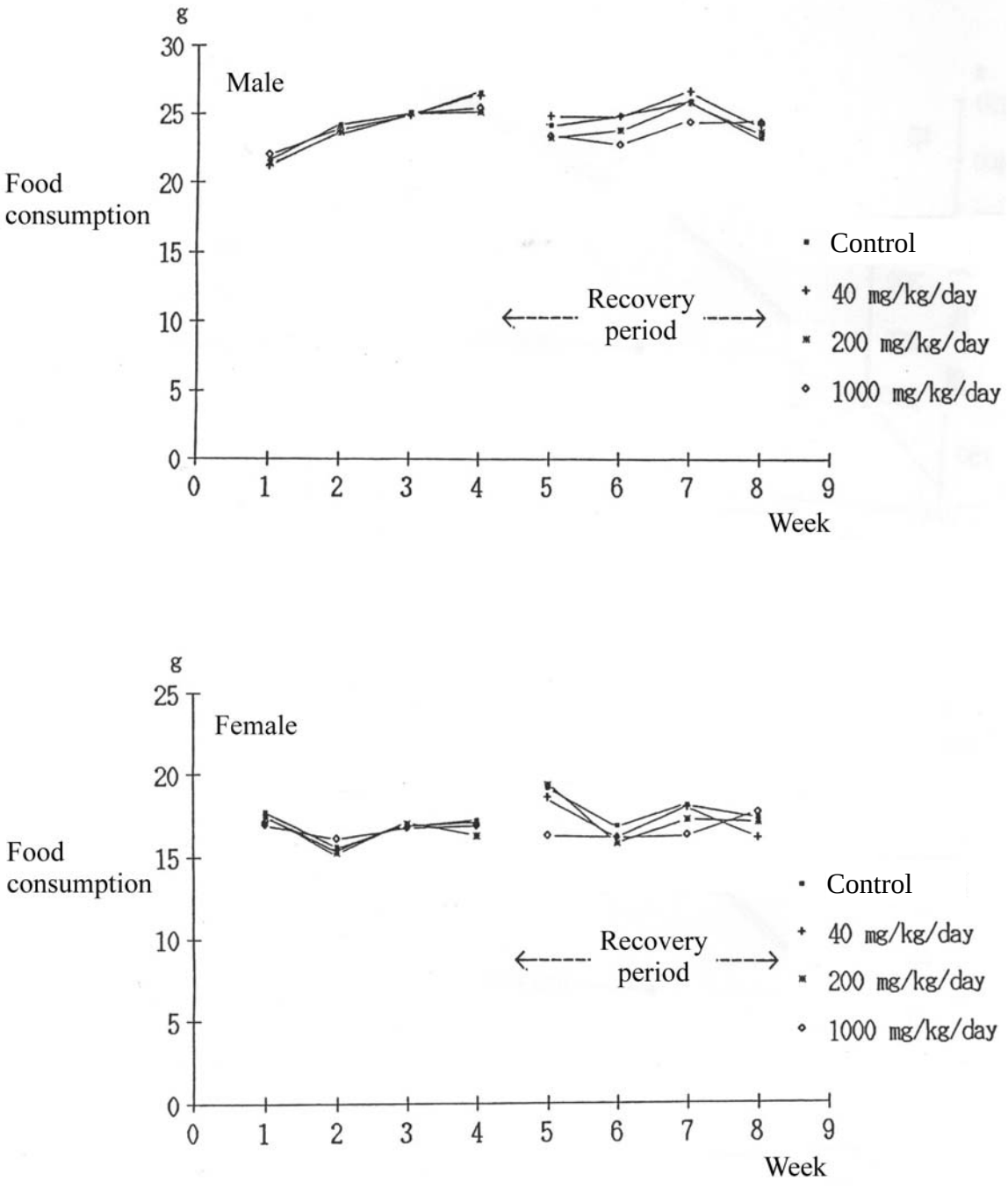

Fig. 2 Food consumption of SD rats during 28 days of daily oral administration of Arbutin and subsequent recovery period

Table 1 Hematology of SD rats after 28 days of daily oral administration of Arbutin

Sex	Dose (mg/kg)	Number of animals	Red blood cell count ($x10^4/mm^3$) Mean S.D.	Hemoglobin (g/dl) Mean S.D.	Hematocrit (%) Mean S.D.	Platelet count ($x10^4/mm^3$) Mean S.D.	White blood cell count ($x10^2/mm^3$) Mean S.D.	Mean red blood cell volume (fl) Mean S.D.	Mean red blood cell hemoglobin (pg) Mean S.D.	Mean red blood cell hemoglobin concentration (%) Mean S.D.	Basophils (%) Mean S.D.	Eosinophils (%) Mean S.D.	Neutrophils (%) Mean S.D.	Lympho-cytes (%) Mean S.D.	Monocytes (%) Mean S.D.	Reticulocytes (%) Mean S.D.
Male	Control	10	764	15.0	45.9	116.1	112	60.1	19.6	32.6	0.0	0.7	9.1	89.1	1.2	3.4
			47	0.6	2.1	4.8	33	2.4	0.9	0.9	0.0	0.6	3.7	4.1	0.4	0.3
	40	10	734	14.8	44.6	112.9	97	60.9	20.2	33.1	0.0	0.6	8.4	89.9	1.1	3.5
			50	0.7	2.3	7.6	47	2.0	1.0	0.7	0.0	0.4	3.1	2.8	0.4	0.4
	200	10	710**	14.6	44.1*	107.2*	80*	62.1*	20.6*	33.1	0.0	0.5	10.1	88.4	1.1	3.6
			32	0.6	1.5	9.3	31	1.3	0.8	0.8	0.0	0.5	3.8	3.3	0.6	0.4
	1000	10	752	14.9	46.2	112.6	86	61.6	19.8	32.3	0.0	0.8	10.9	87.2	1.2	3.5
			55	0.6	3.4	13.4	26	1.9	1.4	2.2	0.0	0.6	5.7	6.0	0.3	0.7
Female	Control	10	746	14.9	44.0	106.5	69	59.2	19.9	33.8	0.0	1.0	8.4	89.5	1.2	2.7
			38	0.7	2.2	11.9	23	1.4	0.6	0.6	0.0	0.7	3.1	3.0	0.4	0.4
	40	10	752	15.2	45.0	110.9	77	59.9	20.3	33.9	0.0	1.1	6.9	91.1	1.1	3.0
			32	0.6	1.6	9.9	30	1.8	0.5	0.6	0.0	0.4	2.4	2.5	0.5	1.0
	200	10	741	15.1	44.5	108.8	83	60.2	20.4	34.0	0.0	0.9	7.8	90.3	1.0	2.7
			27	0.4	1.5	15.7	19	1.5	0.7	0.6	0.0	0.6	3.2	3.5	0.4	0.6
	1000	10	744	14.8	44.1	111.7	91	59.3	19.9	33.6	0.0	0.7	7.4	90.7	1.2	2.8
			51	0.7	2.3	16.7	31	2.0	0.6	0.7	0.0	0.4	2.6	3.0	0.3	0.6

* (Student's t). # (Welch's t): $P < 0.05$
** (Student's t). ## (Welch's t): $P < 0.01$

Table 2 Hematology of SD rats after 28 days of daily oral administration of Arbutin and a 28-day recovery

Sex	Dose (mg/kg)	Number of animals	Red blood cell count ($x10^4/mm^3$) Mean S.D.	Hemoglobin (g/dl) Mean S.D.	Hematocrit (%) Mean S.D.	Platelet count ($x10^4/mm^3$) Mean S.D.	White blood cell count ($x10^2/mm^3$) Mean S.D.	Mean red blood cell volume (fl) Mean S.D.	Mean red blood cell hemoglobin (pg) Mean S.D.	Mean red blood cell hemoglobin concentration (%) Mean S.D.	Basophils (%) Mean S.D.	Eosinophils (%) Mean S.D.	Neutrophils (%) Mean S.D.	Lympho-cytes (%) Mean S.D.	Monocytes (%) Mean S.D.	Reticulocytes (%) Mean S.D.
Male	Control	6	863	15.9	48.4	113.2	116	56.0	18.4	32.7	0.0	0.6	11.8	86.7	1.0	3.0
			40	0.6	2.2	11.6	29	1.7	0.4	0.3	0.0	0.7	2.6	3.4	0.2	0.5
	40	6	832	15.3	46.8	98.5	119	56.5	18.5	32.8	0.0	0.6	11.2	87.4	0.8	2.8
			53	0.5	1.7	12.7	22	3.2	0.7	0.6	0.0	0.5	5.3	5.3	0.2	0.5
	200	6	854	16.0	48.1	106.2	133	56.3	18.8	33.3	0.0	0.9	6.7*	90.1	0.7*	2.8
			28	0.5	1.4	7.6	37	1.5	0.7	0.5	0.0	0.8	4.9	4.0	0.3	0.4
	1000	6	816	15.4	46.3	101.9	114	56.5	18.9*	33.3*	0.0	0.7	8.2*	90.4*	0.8	2.9
			48	1.0	3.1	7.8	54	1.2	0.4	0.5	0.0	0.3	2.3	2.1	0.5	0.3
Female	Control	6	800	15.3	45.3	103.0	105	56.5	19.1	33.7	0.0	1.0	11.1	87.1	0.9	3.2
			42	0.7	1.8	8.6	43	1.4	0.4	0.7	0.0	0.7	2.6	3.5	0.4	0.7
	40	6	784	15.0	44.5	90.1	72	56.5	19.2	33.8	0.0	1.3	8.3	89.4	1.0	2.9
			35	0.6	2.4	18.5	26	1.4	0.4	0.9	0.0	0.7	3.3	3.9	0.4	0.4
	200	6	774	14.8	44.4	102.7	68	57.3	19.2	33.4	0.0	0.8	9.9	88.4	0.9	2.9
			46	0.9	2.4	11.8	14	2.0	0.7	0.5	0.0	0.6	3.4	3.6	0.4	0.7
	1000	6	804	15.2	45.1	97.3	80	56.2	18.9	33.6	0.0	0.7	9.8	88.6	0.9	3.4
			30	0.8	2.0	13.4	29	1.2	0.5	0.6	0.0	0.3	5.5	5.4	0.3	0.5

* (Student's t). # (Welch's t): $P < 0.05$
** (Student's t). ## (Welch's t): $P < 0.01$

Table 3 Serum chemistry of SD rats after 28 days of daily oral administration of Arbutin

Sex	Dose (mg/kg)	Number of animals	Alkaline phosphatase (mU/ml) Mean S.D.	Calcium (mEq/l) Mean S.D.	Total cholesterol (mg/dl) Mean S.D.	Creatinine (mg/dl) Mean S.D.	Glucose (mg/dl) Mean S.D.	Glutamic oxaloacetic transaminase (mU/ml) Mean S.D.	Glutamic pyruvic transaminase (mU/ml) Mean S.D.	Phosphorous (mg/dl) Mean S.D.
Male	Control	10	713	5.0	57	0.3	167	149	38	8.5
			200	0.1	7	0.1	17	33	8	0.3
	40	10	699	5.1	59	0.4	168	147	36	8.7
			95	0.4	10	0.1	23	44	6	0.7
	200	10	685	5.2	60	0.4	175	144	35	8.9
			102	0.2	7	0.1	17	26	7	0.7
	1000	10	590	5.1	62	0.3	172	126	32	8.5
			117	0.1	7	0.1	18	28	6	0.5
Female	Control	10	382	5.2	62	0.4	164	121	26	7.8
			83	0.4	9	0.0	19	22	6	1.4
	40	10	489*	5.4	68	0.4	160	117	28	8.2
			118	0.5	9	0.1	14	21	4	1.2
	200	10	386	5.2	67	0.4	157	125	28	8.1
			88	0.3	9	0.1	20	18	5	0.8
	1000	10	397	5.3	73*	0.3	161	124	26	7.8
			74	0.2	10	0.0	14	60	6	0.8

Sex	Dose (mg/kg)	Number of animals	Total protein (g/dl) Mean S.D.	Triglyceride (mg/dl) Mean S.D.	Blood urea nitrogen (mg/dl) Mean S.D.	Sodium (mEq/l) Mean S.D.	Potassium (mEq/l) Mean S.D.	Chloride (mEq/l) Mean S.D.	Albumin/ Globulin Mean S.D.
Male	Control	10	5.6	115	24	141	5.3	104	1.0
			0.2	45	2	1	0.5	1	0.1
	40	10	5.6	131	25	142	5.1	103	1.0
			0.2	50	4	3	0.5	2	0.1
	200	10	5.6	153	25	143*	5.1	104	1.0
			0.2	47	3	2	0.3	1	0.1
	1000	10	5.7	154	22	142	5.2	102*	1.0
			0.2	67	4	2	0.6	2	0.1
Female	Control	10	5.8	88	31	143	4.9	105	1.0
			0.3	38	24	2	0.4	2	0.1
	40	10	6.0	90	25	143	4.9	105	1.0
			0.4	41	5	2	0.5	2	0.1
	200	10	5.8	76	23	143	4.8	105	0.9
			0.2	34	3	2	0.5	2	0.1
	1000	10	6.0	102	24	143	4.7	105	1.0
			0.4	42	4	2	0.5	1	0.1

* (Student's t). # (Welch's t): $P < 0.05$
** (Student's t). ## (Welch's t): $P < 0.01$

Table 4 Serum chemistry of SD rats after 28 days of daily oral administration of Arbutin and a 28-day recovery

Sex	Dose (mg/kg)	Number of animals	Alkaline phosphatase (mU/ml) Mean S.D.	Calcium (mEq/l) Mean S.D.	Total cholesterol (mg/dl) Mean S.D.	Creatinine (mg/dl) Mean S.D.	Glucose (mg/dl) Mean S.D.	Glutamic oxaloacetic transaminase (mU/ml) Mean S.D.	Glutamic pyruvic transaminase (mU/ml) Mean S.D.	Phosphorous (mg/dl) Mean S.D.
Male	Control	6	400	4.8	57	0.5	167	134	40	7.3
			80	0.3	14	0.1	24	41	6	0.6
	40	6	407	5.0	63	0.5	173	125	35	7.5
			128	0.1	11	0.1	19	11	7	0.6
	200	6	429	5.1*	61	0.4	162	121	41	8.1
			74	0.2	3	0.1	24	21	13	2.0
	1000	6	377	5.0	63	0.5	160	114	37	7.3
			97	0.1	14	0.1	23	32	7	0.4
Female	Control	6	321	5.0	71	0.5	151	117	35	6.4
			88	0.1	17	0.1	20	17	7	0.9
	40	6	281	5.3	68	0.5	149	196	40	7.2
			105	0.4	12	0.1	21	139	10	1.2
	200	6	302	5.1	61	0.5	155	108	35	7.4
			68	0.2	10	0.1	20	19	6	0.8
	1000	6	329	4.9	62	0.5	157	159	33	6.3
			134	0.3	4	0.1	25	109	9	0.8

Sex	Dose (mg/kg)	Number of animals	Total protein (g/dl) Mean S.D.	Triglyceride (mg/dl) Mean S.D.	Blood urea nitrogen (mg/dl) Mean S.D.	Sodium (mEq/l) Mean S.D.	Potassium (mEq/l) Mean S.D.	Chloride (mEq/l) Mean S.D.	Albumin/ Globulin Mean S.D.
Male	Control	6	6.0	124	23	137	4.7	101	0.8
			0.4	49	3	7	0.4	5	0.0
	40	6	6.1	167	23	138	4.9	103	0.9
			0.2	70	1	2	0.4	2	0.1
	200	6	6.3	168	22	138	4.9	102	0.8
			0.1	41	2	1	0.2	2	0.0
	1000	6	6.0	174	24	138	4.8	103	0.9
			0.2	38	2	0	0.3	3	0.1
Female	Control	6	6.3	140	26	139	4.3	104	1.0
			0.2	57	4	3	0.6	3	0.1
	40	6	6.7	110	25	139	4.9	104	1.0
			0.4	68	4	2	1.1	3	0.1
	200	6	6.4	102	23	139	4.3	105	1.0
			0.4	68	2	2	0.3	5	0.1
	1000	6	6.4	87	25	139	4.3	104	1.0
			0.5	59	5	4	0.5	3	0.1

* (Student's t). # (Welch's t): $P < 0.05$
** (Student's t). ## (Welch's t): $P < 0.01$

Table 5 Urinalysis of SD rats after 28 days of daily oral administration of Arbutin

Sex	Dose (mg/kg)	Number of animals	Specific gravity	pH				Protein					Glucose		Ketone bodies		Bilirubin		Occult blood				Nitrite		Urobilinogen	
				6	7	8	9	−	±	+	++	+++	−	+	−	+	−	+	−	±	+	++	−	+	0.1	1
Male	Control	16	1.045 0.009	1	7	8	0	1	0	13	2	0	16	0	16	0	16	0	14	2	0	0	16	0	2	14
	40	16	1.053* 0.009	4	4	8	0	0	1	13	2	0	16	0	16	0	16	0	15	1	0	0	16	0	0	16
	200	16	1.052 0.015	1	5	10	0	0	3	7	6	0	16	0	16	0	16	0	12	4	0	0	16	0	3	13
	1000	16	1.052* 0.010	1	6	9	0	0	0	10	6	0	16	0	16	0	16	0	14	2	0	0	16	0	2	14
Female	Control	16	1.052 0.012	0	4	12	0	0	1	8	7	0	16	0	16	0	16	0	13	2	0	1	16	0	0	16
	40	16	1.056 0.015	6	5	5	0##	0	2	7	6	1	16	0	16	0	16	0	16	0	0	0	16	0	2	14
	200	16	1.055 0.018	5	4	7	0	1	0	9	6	0	16	0	16	0	16	0	15	1	0	0	16	0	5	11
	1000	16	1.052 0.017	0	2	13	1	0	1	5	10	0	16	0	16	0	16	0	15	0	1	0	16	0	2	14

* (Student’s t). # (Mann-Whitney U test): $P < 0.05$
** (Student’s t). ## (Mann-Whitney U test): $P < 0.01$

Table 6 Urinalysis of SD rats after 28 days of daily oral administration of Arbutin and a 28-day recovery

Sex	Dose (mg/kg)	Number of animals	Specific gravity	pH			Protein				Glucose		Ketone bodies		Bilirubin		Occult blood				Nitrite		Urobilinogen	
				6	7	8	−	±	+	++	−	+	−	+	−	+	−	±	+	++	−	+	0.1	1
Male	Control	6	1.046 0.013	0	3	3	0	0	2	4	6	0	6	0	6	0	5	1	0	0	6	0	0	6
	40	6	1.054 0.014	1	4	1	0	0	3	3	6	0	6	0	6	0	4	1	1	0	6	0	0	6
	200	6	1.042 0.015	0	3	3	0	1	3	2	6	0	6	0	6	0	3	3	0	0	6	0	0	6
	1000	6	1.042 0.011	0	3	3	0	0	3	3	6	0	6	0	6	0	4	2	0	0	6	0	1	5
Female	Control	6	1.042 0.010	1	2	3	1	2	3	0	6	0	6	0	6	0	5	1	0	0	6	0	1	5
	40	6	1.041 0.019	0	3	3	3	2	1	0	6	0	6	0	6	0	5	0	0	1	6	0	1	5
	200	6	1.040 0.015	0	1	5	0	2	4	0	6	0	6	0	6	0	6	0	0	0	6	0	2	4
	1000	6	1.034 0.011	0	1	5	0	2	4	0	6	0	6	0	6	0	6	0	0	0	6	0	1	5

* (Student's t). # (Mann-Whitney U test): $P < 0.05$
** (Student's t). ## (Mann-Whitney U test): $P < 0.01$

Table 7 Organ weights of SD rats after 28 days daily oral administration of Arbutin

Sex	Dose (mg/kg)	Number of animals	Brain (g) Mean S.D.	Pituitary gland (mg) Mean S.D.	Salivary gland (g) Mean S.D.	Thymus (g) Mean S.D.	Heart (g) Mean S.D.	Liver (g) Mean S.D.	Spleen (g) Mean S.D.	Kidney (g) Mean S.D.	Adrenal gland (mg) Mean S.D.	Testis (g) Mean S.D.	Prostate (g) Mean S.D.	Ovary (mg) Mean S.D.
Male	Control	10	2.007	10.8	0.566	0.651	1.152	13.639	0.636	2.729	46.4	2.894	0.641	
			0.087	1.6	0.044	0.085	0.093	1.259	0.063	0.265	7.3	0.200	0.105	
	40	10	2.107*	10.5	0.597	0.649	1.106	13.911	0.679	2.715	47.8	2.757	0.671	
			0.076	1.4	0.044	0.117	0.112	1.088	0.168	0.244	5.3	0.163	0.109	
	200	10	2.035	10.6	0.612	0.579	1.134	14.363	0.631	2.765	50.9	2.908	0.726	
			0.071	1.5	0.095	0.153	0.089	1.595	0.131	0.197	5.5	0.276	0.133	
	1000	10	2.046	10.2	0.571	0.613	1.104	14.423	0.616	2.816	46.5	2.860	0.697	
			0.068	1.1	0.063	0.091	0.076	1.719	0.069	0.200	3.9	0.207	0.135	
Female	Control	10	1.859	11.7	0.397	0.474	0.718	8.107	0.427	1.742	59.1			93.9
			0.058	2.6	0.035	0.083	0.067	0.939	0.052	0.089	5.1			14.8
	40	10	1.862	12.4	0.418	0.507	0.758	8.354	0.441	1.828	68.1**			105.2
			0.075	1.6	0.036	0.079	0.062	0.797	0.073	0.143	8.0			17.6
	200	10	1.885	11.6	0.404	0.478	0.722	8.018	0.444	1.723	63.5			94.3
			0.054	2.8	0.050	0.082	0.054	1.044	0.053	0.153	9.7			17.2
	1000	10	1.901	12.2	0.409	0.468	0.750	8.935	0.488	1.842	61.5			91.7
			0.081	2.6	0.042	0.086	0.070	1.110	0.097	0.180	6.3			14.8

* (Student's t). # (Welch's t): $P < 0.05$
** (Student's t). ## (Welch's t): $P < 0.01$

Table 8 Relative organ weights of SD rats after 28 days daily oral administration of Arbutin

Sex	Dose (mg/kg)	Number of animals	Body weight (g) Mean S.D.	Brain (g%) Mean S.D.	Pituitary gland (mg%) Mean S.D.	Salivary gland (g%) Mean S.D.	Thymus (g%) Mean S.D.	Heart (g%) Mean S.D.	Liver (g%) Mean S.D.	Spleen (g%) Mean S.D.	Kidney (g%) Mean S.D.	Adrenal gland (mg%) Mean S.D.	Testis (g%) Mean S.D.	Prostate (g%) Mean S.D.	Ovary (mg%) Mean S.D.
Male	Control	10	329.1	0.613	3.28	0.173	0.199	0.351	4.144	0.193	0.830	14.08	0.881	0.195	
			25.5	0.047	0.39	0.016	0.030	0.026	0.185	0.018	0.056	1.85	0.051	0.033	
	40	10	332.9	0.635	3.13	0.179	0.196	0.333	4.178	0.203	0.815	14.35	0.832	0.202	
			21.2	0.040	0.26	0.011	0.038	0.030	0.180	0.045	0.041	1.09	0.083	0.032	
	200	10	335.1	0.609	3.17	0.182	0.173	0.339	4.278	0.188	0.827	15.16	0.870	0.217	
			23.8	0.028	0.41	0.021	0.045	0.025	0.238	0.033	0.054	1.05	0.088	0.040	
	1000	10	331.7	0.619	3.06	0.172	0.185	0.333	4.339	0.186	0.850	14.07	0.865	0.210	
			21.4	0.040	0.28	0.014	0.027	0.006	0.306	0.018	0.051	1.41	0.083	0.036	
Female	Control	10	203.6	0.916	5.78	0.195	0.233	0.353	3.979	0.210	0.858	29.09			46.24
			12.1	0.056	1.47	0.015	0.039	0.023	0.356	0.026	0.050	2.76			7.85
	40	10	204.3	0.913	6.08	0.205	0.248	0.371	4.087	0.216	0.895	33.37*			51.77
			9.5	0.056	0.71	0.015	0.036	0.023	0.301	0.034	0.058	3.85			10.37
	200	10	198.6	0.952	5.80	0.203	0.241	0.364	4.027	0.223	0.868	31.96			47.36
			13.9	0.052	1.14	0.018	0.039	0.012	0.305	0.021	0.063	4.09			7.25
	1000	10	215.0	0.889	5.70	0.191	0.219	0.349	4.148	0.227	0.858	28.66			43.10
			20.0	0.064	1.10	0.017	0.039	0.023	0.219	0.037	0.053	2.21			9.40

* (Student's t). # (Welch's t): $P < 0.05$
** (Student's t). ## (Welch's t): $P < 0.01$

Table 9 Organ weights of SD rats after 28 days of daily oral administration of Arbutin and a 28-day recovery

Sex	Dose (mg/kg)	Number of animals	Brain (g) Mean S.D.	Pituitary gland (mg) Mean S.D.	Salivary gland (g) Mean S.D.	Thymus (g) Mean S.D.	Heart (g) Mean S.D.	Liver (g) Mean S.D.	Spleen (g) Mean S.D.	Kidney (g) Mean S.D.	Adrenal gland (mg) Mean S.D.	Testis (g) Mean S.D.	Prostate (g) Mean S.D.	Ovary (mg) Mean S.D.
Male	Control	6	2.094	12.5	0.691	0.447	1.301	15.679	0.721	3.129	53.8	3.262	1.027	
			0.083	2.0	0.062	0.056	0.104	1.572	0.057	0.295	8.3	0.151	0.081	
	40	6	2.139	12.3	0.650	0.481	1.338	16.237	0.762	2.992	56.1	3.120	0.983	
			0.031	1.6	0.060	0.100	0.134	1.470	0.109	0.310	11.1	0.253	0.165	
	200	6	2.096	12.1	0.664	0.469	1.372	16.319	0.773	3.056	50.0	3.140	1.001	
			0.112	2.8	0.076	0.154	0.130	2.122	0.113	0.234	8.4	0.292	0.176	
	1000	6	2.190	11.3	0.684	0.467	1.310	16.301	0.776	3.201	53.2	3.197	0.953	
			0.094	1.4	0.067	0.080	0.101	1.448	0.108	0.287	8.2	0.332	0.309	
Female	Control	6	2.026	12.7	0.411	0.359	0.839	8.914	0.506	1.891	72.1			95.1
			0.049	1.1	0.028	0.081	0.087	0.853	0.062	0.223	7.5			14.6
	40	6	2.005	13.5	0.454	0.355	0.846	9.335	0.505	1.838	72.0			102.5
			0.075	2.3	0.063	0.094	0.099	1.953	0.044	0.082	8.6			16.7
	200	6	1.909**	13.2	0.439	0.332	0.806	8.535	0.500	1.753	65.4			82.5
			0.070	1.0	0.035	0.024	0.072	0.883	0.078	0.155	7.7			16.3
	1000	6	1.966	12.8	0.419	0.322	0.793	8.342	0.443	1.802	62.5*			76.8*
			0.059	1.0	0.035	0.108	0.037	0.671	0.050	0.143	3.9			11.8

* (Student's t). # (Welch's t): $P < 0.05$
** (Student's t). ## (Welch's t): $P < 0.01$

Table 10 Relative organ weights of SD rats after 28 days of daily oral administration of Arbutin and a 28-day recovery

Sex	Dose (mg/kg)	Number of animals	Body weight (g) Mean S.D.	Brain (g%) Mean S.D.	Pituitary gland (mg%) Mean S.D.	Salivary gland (g%) Mean S.D.	Thymus (g%) Mean S.D.	Heart (g%) Mean S.D.	Liver (g%) Mean S.D.	Spleen (g%) Mean S.D.	Kidney (g%) Mean S.D.	Adrenal gland (mg%) Mean S.D.	Testis (g%) Mean S.D.	Prostate (g%) Mean S.D.	Ovary (mg%) Mean S.D.
Male	Control	6	432.6	0.486	2.89	0.160	0.103	0.301	3.620	0.167	0.724	12.52	0.755	0.239	
			28.7	0.037	0.38	0.011	0.010	0.010	0.194	0.013	0.054	2.25	0.029	0.030	
	40	6	446.1	0.481	2.75	0.146	0.109	0.300	3.635	0.172	0.669	12.61	0.699**	0.219	
			25.5	0.023	0.37	0.012	0.028	0.025	0.153	0.028	0.033	2.51	0.027	0.027	
	200	6	439.4	0.479	2.74	0.151	0.106	0.313	3.705	0.176	0.697	11.38	0.715*	0.228	
			36.0	0.035	0.48	0.016	0.029	0.030	0.236	0.022	0.050	1.61	0.029	0.039	
	1000	6	437.9	0.502	2.59	0.157	0.107	0.299	3.723	0.177	0.732	12.11	0.730	0.215	
			29.8	0.044	0.28	0.022	0.021	0.012	0.218	0.020	0.059	1.41	0.058	0.061	
Female	Control	6	264.7	0.771	4.83	0.156	0.137	0.318	3.379	0.191	0.717	27.56			36.03
			26.6	0.067	0.51	0.016	0.034	0.017	0.252	0.014	0.084	4.65			4.88
	40	6	257.5	0.790	5.26	0.177	0.130	0.330	3.599	0.198	0.721	28.26			40.29
			35.1	0.106	0.67	0.019	0.034	0.030	0.294	0.018	0.062	4.36			8.22
	200	6	248.6	0.771	5.33	0.177*	0.134	0.325	3.433	0.201	0.705	26.27			33.35
			16.1	0.052	0.57	0.015	0.012	0.028	0.275	0.032	0.027	1.96			7.47
	1000	6	242.3	0.814	5.27	0.173	0.132	0.328	3.446	0.184	0.744	25.89			31.82
			12.6	0.056	0.40	0.010	0.039	0.022	0.275	0.028	0.055	2.61			5.62

* (Student's t). # (Welch's t): $P < 0.05$
** (Student's t). ## (Welch's t): $P < 0.01$

90-Day Repeated-dose Percutaneous Toxicity Study of Arbutin in Rats

90-Day Percutaneous Repeated-dose Toxicity Study of Arbutin in Rats

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1. Introduction

This study evaluated percutaneous repeated-dose toxicity of Arbutin.

2. Administration Period

The first administration: Feb. 27, 1986
The final administration: May 29, 1986

3. Materials and Methods

3.1 Animals and Housing Conditions

Female and male SPF Sprague-Dawley (SD) rats (Crj:CD, Charles River Japan Inc.) were purchased at 4 weeks of age. After a one-week acclimation period, animals appearing normal were divided into groups with equal average body weight. Body weight was 136 to 156 g in males and 106 to 130 g in females at the start of the study.

Animals were housed throughout the acclimation and test periods in a barrier facility. Temperature and humidity of the animal quarters were maintained at 23 ± 2ºC and 55 ± 5% relative humidity, respectively, with an air exchange frequency of 32 times/hour and a light cycle of 12 hours. The rats were housed individually in suspended wire-mesh metal cages. They were fed laboratory chow (radiation-sterilized, NMFR: Oriental Yeast Co., Ltd.) and tap water (ultraviolet ray and microfilter-treated) *ad libitum*.

3.2 Test substance

Arbutin (Lot a, Nippon Fine Chemical Co., Ltd.) was used as the test substance.

3.2.1 Preparation of test substance

The test substance was dissolved in 50% ethanol for administration. Solutions were prepared fresh once per week. Aliquots of prepared test substance were dispensed into amber screw cap bottles and stored at room temperature.

3.3 Dosage groups and administration method

A pilot 14-day toxicity study was conducted to determine doses for this study.

The maximum feasible dosage was 928 mg/kg in the acute toxicity study. No toxicity was observed at 928 mg/kg in the acute toxicity study. The concentration used in the pilot 14-day toxicity study was the same as that used in the acute toxicity study for the following reason. Solubility of the test substance in a 50% ethanol vehicle increases with temperature, and is 30% (w/w) at 37ºC (body temperature in rats). Thus, 30% (w/w), 15% (w/w), and vehicle control were chosen as dose formulations. The maximum dose volume was 3 mL/kg in the acute toxicity study. It was noted that the application area on the back in rats did not increase with body weight, and the maximum dose volume was limited in the pilot study to 2 mL/kg in consideration of daily administration. In the pilot study, no test article-related abnormality was observed in clinical signs, body weights, food consumption, organ weights or necropsy findings. The maximum dosage in the pilot study was established as the maximum dosage for the 90-day repeated-dose toxicity study.

Animals were divided into four groups at dose levels of 618 mg/kg (30%, w/w, specific gravity 1.03), 294 mg/kg (15%, w/w, specific gravity 0.98), 56 mg/kg (3%, w/w, specific gravity 0.94) and vehicle control group. Dose volume was 2 mL/kg in all treated groups. To assess effects of the vehicle on skin at the application site, a sham treated (fur clipping only) group was included as a control. For this control group, only necropsy and histopathological examination of the clipped skin were conducted. Each group consisted of 10 female and 10 male rats.

Prepared test substance or vehicle (50% aqueous ethanol solution) was applied to the dorsal skin (clipped of fur) 6 days per week for 90 days. Fur clipping was performed once per week on Tuesdays.

3.4 Observations

3.4.1 Clinical signs, body weight and food consumption

Clinical signs were observed at the time the test substance or vehicle was applied. Body weight and 24-hour food consumption were measured once per week during the dosing period.

3.4.2 Hematology

Blood for hematology was collected under ether anesthesia from the abdominal aorta. Edetate dipotassium was used as the anticoagulant. Hematology included red blood cell count, white blood cell count, platelet count (electronically enumerated), hemoglobin (cyanmethemoglobin method), mean red blood cell volume (MCV), mean red blood cell hemoglobin (MCH), and mean red blood cell hemoglobin concentration (MCHC) by an automatic hemocytometer (Model CC-180A, Toa Iyo Denshi Co., Ltd.). The hematocrit was measured by capillary centrifugation. Reticulocyte count (new methylene blue stain) and

white blood cell differential count (May-Giemsa staining) were also performed.

3.4.3 Serum chemistry

Blood collected under ether anesthesia from the abdominal aorta was allowed to clot for 30 to 40 minutes at room temperature, and then centrifuged (at 3000 rpm, 15 min.). Serum was sent to Toukuri Laboratory to measure the following analytes: total protein (Biuret method), A/G ratio (Biuret method, BCG method), GOT, GPT (UV method), ALP (GSCC compliance), total cholesterol (enzymatic method), triglyceride (enzymatic method), blood urea nitrogen (urease-GLDH method), creatinine (Jaffe method), glucose (enzymatic method), Na^{+}, K^{+}, and Cl^{-} (electrode method), Ca^{++} (O-CPC method), and inorganic phosphorous (enzymatic method). An autoanalyzer (Hitachi Model 736) was used for serum chemistry.

3.4.4 Urinalysis

Urinalysis was conducted during the 13th week of dosing using fresh urine collected by abdominal compression. Urinalysis test paper (Miles-Sankyo, N-multi-sticks III) was used to measure pH, protein, glucose, ketone bodies, bilirubin, occult blood, nitrite, and urobilinogen.

3.4.5 Pathology

Animals were exsanguinated prior to necropsy.

The following organ weights were measured: brain, pituitary gland, salivary gland, thymus, heart, liver, spleen, kidney, adrenal gland, prostate, testis, and ovary. Relative organ weight was calculated by dividing by body weight on the day of necropsy. In addition to measured organs, the following tissues were fixed in 10% buffered formalin: skin (application site), parotid gland, trachea, thyroid gland, tongue, lung, esophagus, stomach, small intestine, large intestine, mesenteric lymph nodes, pancreas urinary, bladder, seminal vesicle, uterus, vagina, Harderian gland, eye, femur, and spinal cord. Tissues and organ specimens were imbedded in paraffin, blocked and sectioned, and stained with hematoxylin and eosin for histopathology.

3.5 Statistical methods

Quantitative parameters were evaluated by Student’s t-test (normal distribution) or Welch’s modification of the Student’s t-test (skewed distribution). The rank-sum test (Mann-Whitney U test) was used for semi-quantitative urinalysis values.

4. Results

4.1 Clinical signs

All animals survived and no abnormalities were recorded in any group during the observation period.

4.2 Body weight

Fig. 1 presents body weight during the observation period.

Body weight was reduced between the 6th and 8th weeks in males at 56 mg/kg. In females given the test substance, body weight showed a trend similar to that of the vehicle control group.

4.3 Food consumption

Fig. 2 shows food consumption.

Food consumption was variable in all groups. Statistically significant differences from the vehicle control group were sporadically observed. A remarkable increase was observed in the female vehicle control group in the 7th week.

4.4 Hematology

Table 1 shows results of the hematological examination.

No statistically significant difference was observed from the vehicle control group in males. An increase in monocyte ratio was observed in females at 618 mg/kg.

4.5 Serum chemistry

Table 2 indicates the result of serum chemistry.

No statistically significant difference from the vehicle control group was observed in males. A decrease in Ca^{++} was observed in females at 294 mg/kg.

4.6 Urinalysis

Table 3 shows the result of urinalysis. There was no test substance-related change in any parameter.

4.7 Necropsy findings

There were no remarkable findings at necropsy in either sex at any dose level.

4.8 Organ weights

Tables 4 and 5 show absolute and relative organ weights at the end of the 90-day application period. A decrease in absolute weight of pituitary gland was observed in males at 294 mg/kg and increases in relative weights of thymus, spleen and adrenal were observed at 56 mg/kg. In females, decreases in absolute and relative weights of pituitary gland and thymus were observed

in the 294 mg/kg group, and a decrease in relative weight of thymus was seen in the 618 mg/kg group.

4.9 Histopathology

No histopathological changes associated with the test substance were observed. No changes were found in the cuticle and corium of the skin of the application site. In dosed groups, including the vehicle control group, findings included sporadic small granuloma in the liver, fibrous cardiac muscle, and minor infiltrations of small round cells in the kidney, prostate and Harderian gland.

5. Discussion

Body weight in males at 56 mg/kg was reduced between the 6th and 8th weeks. Body weight in males at 294 and 618 mg/kg showed the same trend as the vehicle control group. No dose-dependency was observed and the decrease is considered within the normal range of variation.

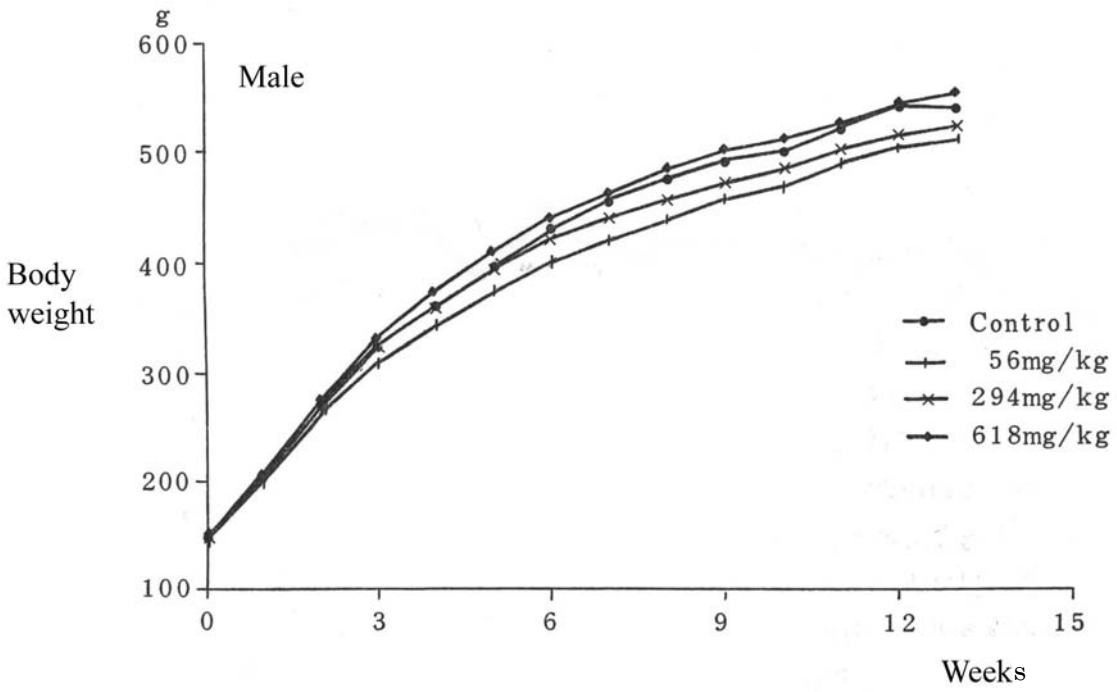
Food consumption was variable in every group throughout the study period, without any obvious dose relationship. A remarkable increase in food consumption was observed in the female vehicle control group during the 7th week. This increase in food consumption was attributed to an animal that raked out food from the hopper into the cage. This animal had not raked food out until the 6th week, and the raking behavior stopped in the 8th week.

There was an increase in monocyte ratio in females at 618 mg/kg and a decrease of Ca^{++} in females at 294 mg/kg, but these are regarded as being within normal physiological variation.

No abnormalities were observed in clinical signs and in urinalysis. No abnormalities were observed in histolopathological examination corresponding to any change in organ weight. No test substance-related abnormalities were observed at necropsy or histopathological examination.

6. Conclusion

Subacute percutaneous toxicity of Arbutin was evaluated by repeated application to the back of rats at 56, 294 and 618 mg/kg/day (the maximum technically applicable dose) for 90 days. No test substance-related changes were observed in any measure in any group of animals. Therefore, it is considered that the “no observed effect” level of Arbutin is at least 618 mg/kg/day.



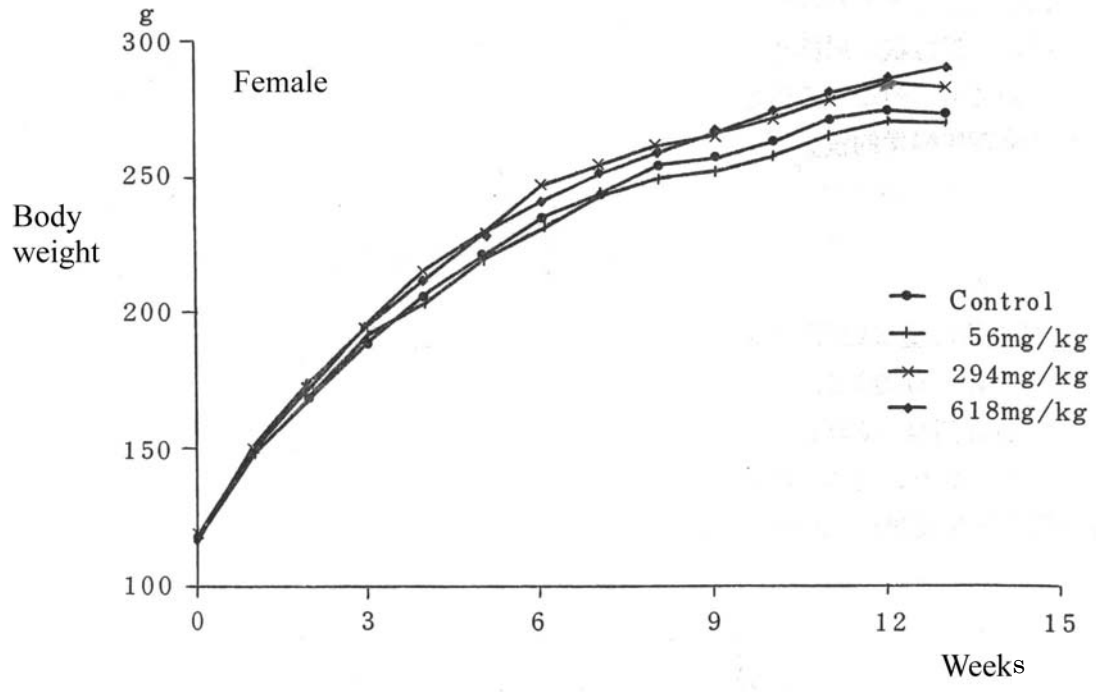


Fig. 1 Body weight of SD rats treated percutaneously with Arbutin for 90 days.

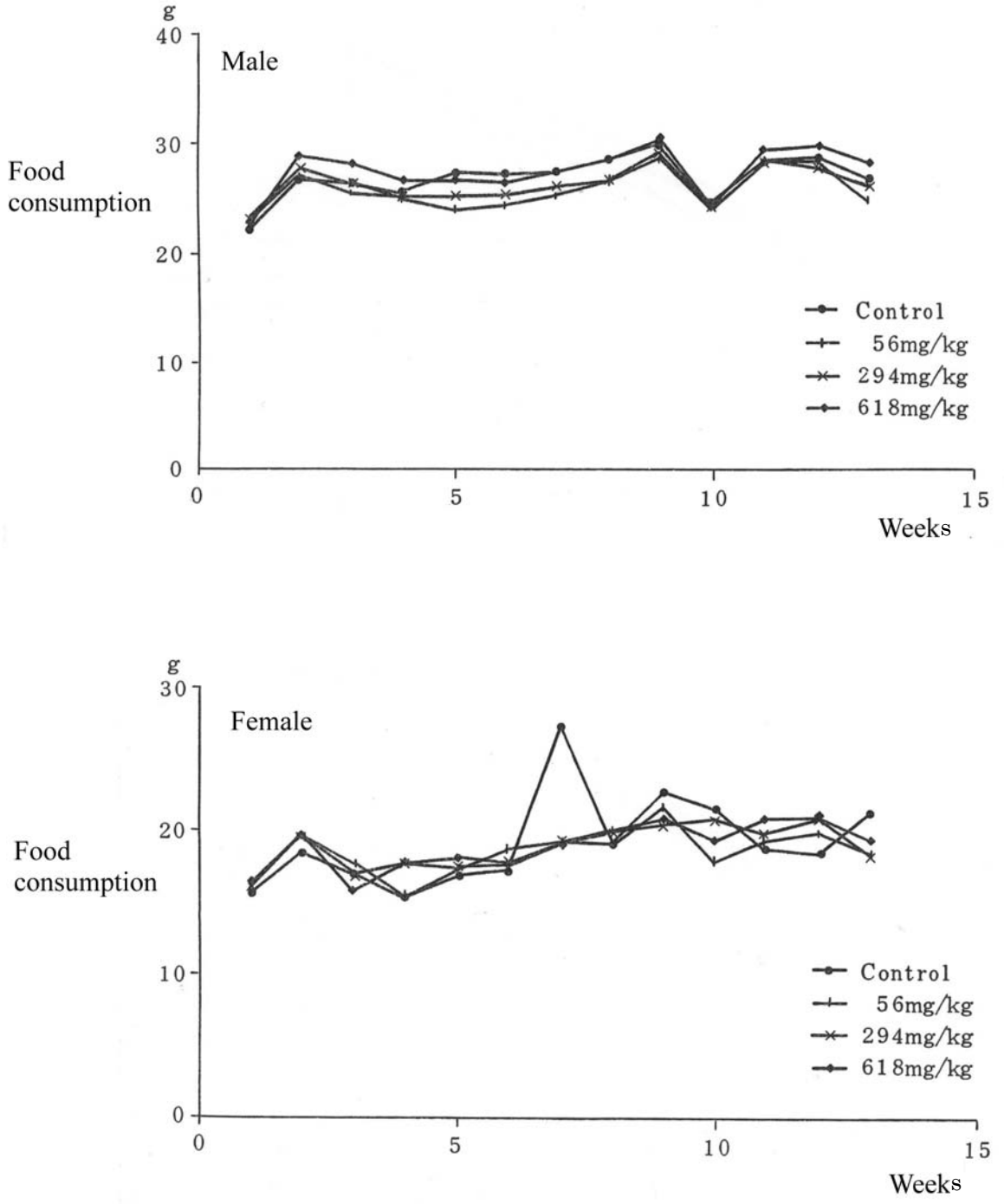


Fig. 2 Food consumption of SD rats treated percutaneously with Arbutin for 90 days.

Table 1 Hematology of SD rats treated percutaneously with Arbutin for 90 days

Sex	Group	Dose (mg/kg)	Red blood cell count			Hemoglobin			Hematocrit			Mean red blood cell hemoglobin			Mean red blood cell volume		
			Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals
			(10^4/mm^3)			(g/dl)			(%)			(pg)			(fl)		
Male	1	Control	867	20	10	15.0	0.3	10	48.0	2.4	5	17.3	0.5	10	55.0	2.4	5
	2	56	855	47	10	15.0	0.7	10	45.5	4.2	5	17.5	0.7	10	53.0	3.5	5
	3	294	862	48	9	15.1	0.6	9	48.6	3.2	6	17.5	0.5	9	57.0	3.9	6
	4	618	873	61	10	15.2	0.4	10	47.3	3.3	6	17.5	1.0	10	54.3	4.4	6
Female	1	Control	803	34	10	14.9	0.5	10	46.4	2.0	6	18.6	0.6	10	57.7	2.1	6
	2	56	811	39	10	15.1	0.9	10	46.3	2.7	6	18.7	0.3	10	57.3	2.6	6
	3	294	815	41	10	15.3	0.6	10	47.0	2.1	6	18.7	0.5	10	58.8	4.4	6
	4	618	798	52	10	15.0	0.7	10	44.7	2.4	6	18.9	0.8	10	58.7	3.7	6

Sex	Group	Dose (mg/kg)	Mean red blood cell hemoglobin concentration			Platelet count			White blood cell count		
			Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals
			(%)			(10^4/mm^3)			(10^2/mm^3)		
Male	1	Control	31.5	1.5	5	111.9	14.9	10	91	35	10
	2	56	32.5	1.7	5	117.0	15.5	10	69	21	10
	3	294	30.9	1.8	6	107.9	9.2	9	64	27	9
	4	618	32.1	2.3	6	117.9	11.1	10	86	34	10
Female	1	Control	32.3	1.3	6	103.8	12.8	10	53	28	10
	2	56	32.7	1.5	6	103.8	13.3	10	38	16	10
	3	294	32.2	2.1	6	109.6	11.0	10	48	21	10
	4	618	33.1	2.2	6	99.7	11.3	10	43	25	10

(Table 1 continued)

Sex	Group	Dose (mg/kg)	Reticulocytes			Basophils			Eosinophils			Neutrophils			Lymphocytes			Monocytes		
			Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals
			(%)			(%)			(%)			(%)			(%)			(%)		
Male	1	Control	2.4	0.7	10	0.0	0.0	10	1.0	0.6	10	10.7	4.3	10	87.2	4.8	10	1.2	0.7	10
	2	56	2.4	0.8	10	0.0	0.0	10	1.1	0.7	10	10.7	1.2	10	87.4	1.8	10	0.9	0.5	10
	3	294	2.3	0.8	9	0.0	0.0	9	1.0	0.9	9	11.8	5.7	9	86.3	5.6	9	1.0	0.4	9
	4	618	2.5	0.7	10	0.0	0.0	10	1.0	0.6	10	10.2	3.0	10	87.9	3.1	10	1.0	0.4	10
Female	1	Control	2.0	0.7	10	0.0	0.0	10	1.8	1.2	10	11.0	4.3	10	86.6	4.8	10	0.7	0.4	10
	2	56	2.2	0.9	10	0.0	0.0	10	1.8	1.1	10	15.5	10.4	10	81.8	11.1	10	1.1	0.7	10
	3	294	2.3	0.5	10	0.0	0.0	10	1.4	0.6	10	12.4	5.0	10	85.3	4.9	10	1.0	0.4	10
	4	618	2.2	0.6	10	0.0	0.0	10	1.3	0.8	10	13.2	6.1	10	84.4	6.5	10	1.2*	0.6	10

* (Student’s t). # (Welch’s t): $P < 0.05$
** (Student’s t). ## (Welch’s t): $P < 0.01$

Table 2 Serum chemistry of SD rats treated percutaneously with Arbutin for 90 days

Sex	Group	Dose (mg/kg)	Glutamic oxaloacetic transaminase			Glutamic pyruvic transaminase			Alkaline phosphatase			Total cholesterol			Triglyceride		
			Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals
			(mU/mL serum)			(mU/mL serum)			(mU/mL serum)			(mg/dl serum)			(mg/dl serum)		
Male	1	Control	119	21	10	31	6	10	352	79	10	67	8	10	166	63	10
	2	56	128	31	10	35	10	10	414	92	10	66	15	10	127	37	10
	3	294	132	41	10	32	6	10	420	78	10	74	12	10	164	45	10
	4	618	130	21	10	36	7	10	353	64	10	76	20	10	163	52	10
Female	1	Control	122	30	10	37	10	10	287	134	10	69	7	10	77	53	10
	2	56	138	32	10	44	24	10	303	99	10	65	11	10	66	25	10
	3	294	126	40	10	31	9	10	250	58	10	68	11	10	81	59	10
	4	618	131	26	10	37	13	10	273	84	10	70	11	10	99	51	10

Sex	Group	Dose (mg/kg)	Total protein			Albumin/Globulin			Glucose			Blood urea nitrogen			Creatinine		
			Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals
			(g/dl serum)			(Serum)			(mg/dl serum)			(mg/dl serum)			(mg/dl serum)		
Male	1	Control	6.4	0.3	10	0.9	0.1	10	168	18	10	23	3	10	0.4	0.0	10
	2	56	6.4	0.3	10	0.9	0.0	10	187	37	10	25	2	10	0.5	0.1	10
	3	294	6.4	0.3	10	0.9	0.1	10	179	22	10	24	4	10	0.5	0.1	10
	4	618	6.5	0.2	10	0.9	0.1	10	195*	27	10	23	3	10	0.4	0.1	10
Female	1	Control	6.5	0.3	10	1.0	0.1	10	177	20	10	25	5	10	0.5	0.1	10
	2	56	6.6	0.4	10	1.0	0.1	10	169	19	10	23	3	10	0.5	0.1	10
	3	294	6.4	0.3	10	1.0	0.1	10	170	18	10	22	3	10	0.5	0.1	10
	4	618	6.6	0.3	10	1.1	0.1	10	173	21	10	24	4	10	0.4	0.0	10

(Table 2 continued)

Sex	Group	Dose (mg/kg)	Phosphorous			Sodium			Potassium			Calcium			Chloride		
			Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals
			(mg/dl serum)			(mEq/l serum)			(mEq/l serum)			(mEq/l serum)			(mEq/l serum)		
Male	1	Control	6.9	0.5	10	140	1	10	4.9	0.3	10	5.0	0.2	10	103	1	10
	2	56	6.6	0.7	10	139	2	10	4.9	0.3	10	5.0	0.1	10	102	2	10
	3	294	6.9	0.6	10	140	3	10	4.9	0.4	10	4.9	0.1	10	102	3	10
	4	618	6.7	0.6	10	140	1	10	4.9	0.3	10	5.0	0.1	10	103	1	10
Female	1	Control	6.3	1.3	10	140	2	10	4.3	0.4	10	5.0	0.1	10	104	1	10
	2	56	7.2	1.9	10	141	3	10	4.7	0.7	10	5.1	0.3	10	104	1	10
	3	294	6.0	0.8	10	138	2	10	4.4	0.4	10	4.9*	0.1	10	103	2	10
	4	618	5.8	0.7	10	139	2	10	4.4	0.2	10	5.0	0.2	10	103	2	10

* (Student’s t). # (Welch’s t): $P < 0.05$
** (Student’s t). ## (Welch’s t): $P < 0.01$

Table 3 Urinalysis of SD rats treated percutaneously with Arbutin for 90 days

Sex	Group	Dose (mg/kg)	pH			Protein					Glucose		Ketone bodies		Bilirubin		Occult blood				Nitrite		Urobilinogen	
			6	7	8	−	±	+	++	+++	−	±	−	+	−	+	−	±	+	++	−	+	0.1	1
Male	1	Control	1	9	0	0	0	6	3	0	10	0	10	0	10	0	7	1	1	1	10	0	1	9
	2	56	1	7	2	0	0	6	3	1	10	0	10	0	10	0	5	4	1	0	10	0	0	10
	3	294	2	7	1	0	1	4	5	0	10	0	10	0	10	0	6	4	0	0	10	0	0	10
	4	618	0	7	3	0	0	7	2	1	10	0	10	0	10	0	8	1	1	0	10	0	0	10
Female	1	Control	3	5	2	0	5	4	1	0	10	0	10	0	10	0	9	1	0	0	10	0	2	8
	2	56	7	1	2	0	8	1	1	0	10	0	10	0	10	0	9	1	0	0	10	0	4	6
	3	294	6	2	2	0	7	3	0	0	10	0	10	0	10	0	8	2	0	0	10	0	1	9
	4	618	3	6	1	0	4	6	0	0	10	0	10	0	10	0	9	1	0	0	10	0	2	8

* (Student's t). # (Mann-Whitney U test): $P < 0.05$
** (Student's t). ## (Mann-Whitney U test): $P < 0.01$

Table 4 Absolute organ weights of SD rats treated percutaneously with Arbutin for 90 days

Sex	Group	Dose (mg/kg)	Body weight			Brain			Pituitary gland			Thymus			Heart			Salivary gland		
			Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals
			(g)			(g)			(mg)			(g)			(g)			(g)		
Male	1	Control	540.57	39.83	10	2.152	0.174	10	14.2	1.2	10	0.269	0.085	10	1.361	0.102	10	0.697	0.075	10
	2	56	509.79	59.06	10	2.008	0.213	10	12.3	2.9	10	0.321	0.063	10	1.324	0.127	10	0.698	0.060	10
	3	294	524.58	29.43	10	2.149	0.073	10	12.6*	1.6	10	0.293	0.055	10	1.379	0.059	10	0.719	0.067	10
	4	618	552.74	33.12	10	2.148	0.222	10	13.4	1.4	10	0.319	0.095	10	1.417	0.104	10	0.698	0.073	10
Female	1	Control	274.63	23.16	10	1.879	0.181	10	14.2	1.5	10	0.304	0.063	10	0.825	0.057	10	0.447	0.056	10
	2	56	271.46	23.09	10	1.876	0.143	10	13.1	3.2	10	0.254	0.075	10	0.812	0.080	10	0.463	0.057	10
	3	294	284.40	28.55	10	1.902	0.151	10	12.0**	1.8	10	0.250*	0.036	10	0.811	0.058	10	0.464	0.032	10
	4	618	291.52	29.73	10	1.731	0.467	10	14.2	2.4	10	0.264	0.043	10	0.959	0.247	10	0.471	0.061	10

Sex	Group	Dose (mg/kg)	Liver			Spleen			Adrenal gland			Kidney			Testis, Ovary			Prostate		
			Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animal	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals
			(g)			(g)			(mg)			(g)			M (g)	F (mg)		(g)		
Male	1	Control	18.559	2.659	10	0.710	0.088	10	58.5	6.1	10	3.522	0.223	10	3.124	0.157	10	1.33	0.27	10
	2	56	18.335	2.856	10	0.760	0.085	10	61.1	7.9	10	3.362	0.420	10	3.156	0.148	10	1.14	0.29	10
	3	294	18.093	1.711	10	0.744	0.083	10	57.4	7.3	10	3.587	0.413	10	3.194	0.240	10	1.22	0.25	10
	4	618	19.416	2.232	10	0.764	0.108	10	60.5	8.2	10	3.620	0.358	10	3.178	0.273	10	1.20	0.25	10
Female	1	Control	8.886	1.008	10	0.478	0.076	10	70.9	9.9	10	1.912	0.141	10	110.9	23.2	10			
	2	56	8.856	1.193	10	0.450	0.070	10	67.0	9.6	10	1.894	0.185	10	102.3	23.6	10			
	3	294	8.542	1.150	10	0.470	0.059	10	70.4	6.7	10	1.890	0.131	10	105.7	17.6	10			
	4	618	9.441	0.815	10	0.591	0.375	10	69.7	10.1	10	2.045	0.287	10	113.2	16.8	10			

* (Student's t). # (Welch's t): $P < 0.05$
** (Student's t). ## (Welch's t): $P < 0.01$

Table 5 Relative organ weights of SD rats treated percutaneously with Arbutin for 90 days

Sex	Group	Dose (mg/kg)	Body weight			Brain			Pituitary gland			Thymus			Heart			Salivary gland		
			Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals
			(g)			(g %)			(mg %)			(g %)			(g %)			(g %)		
Male	1	Control	540.57	39.83	10	0.401	0.049	10	2.64	0.24	10	0.050	0.016	10	0.253	0.023	10	0.129	0.014	10
	2	56	509.79	59.06	10	0.395	0.034	10	2.41	0.44	10	0.063*	0.008	10	0.260	0.012	10	0.138	0.017	10
	3	294	524.58	29.43	10	0.411	0.025	10	2.41	0.30	10	0.056	0.010	10	0.264	0.016	10	0.137	0.013	10
	4	618	552.74	33.12	10	0.390	0.052	10	2.43	0.32	10	0.058	0.016	10	0.257	0.025	10	0.127	0.014	10
Female	1	Control	274.63	23.16	10	0.687	0.074	10	5.22	0.73	10	0.111	0.021	10	0.302	0.026	10	0.163	0.020	10
	2	56	271.46	23.09	10	0.693	0.047	10	4.87	1.53	10	0.093	0.025	10	0.300	0.030	10	0.172	0.027	10
	3	294	284.40	28.55	10	0.674	0.080	10	4.27*	0.89	10	0.089*	0.015	10	0.286	0.016	10	0.164	0.017	10
	4	618	291.52	29.73	10	0.600	0.171	10	4.91	0.92	10	0.091*	0.019	10	0.329	0.075	10	0.163	0.025	10

Sex	Group	Dose (mg/kg)	Liver			Spleen			Adrenal gland			Kidney			Testis, Ovary			Prostate		
			Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals	Mean	S.D.	Number of animals
			(g %)			(g %)			(mg %)			(g %)			M (g %)	F (mg %)		(g %)		
Male	1	Control	3.424	0.317	10	0.131	0.014	10	10.81	0.67	10	0.654	0.056	10	0.580	0.035	10	0.25	0.05	10
	2	56	3.586	0.238	10	0.150**	0.013	10	12.05#	1.54	10	0.661	0.051	10	0.626	0.072	10	0.23	0.06	10
	3	294	3.446	0.208	10	0.142	0.015	10	10.99	1.61	10	0.684	0.063	10	0.611	0.059	10	0.23	0.05	10
	4	618	3.509	0.312	10	0.138	0.018	10	11.00	1.70	10	0.656	0.063	10	0.579	0.076	10	0.22	0.04	10
Female	1	Control	3.243	0.326	10	0.175	0.030	10	25.97	4.08	10	0.700	0.071	10	40.67	9.74	10			
	2	56	3.264	0.353	10	0.167	0.027	10	24.81	3.88	10	0.702	0.092	10	37.52	6.79	10			
	3	294	3.004	0.281	10	0.166	0.023	10	24.91	2.83	10	0.668	0.048	10	37.63	7.71	10			
	4	618	3.252	0.262	10	0.202	0.124	10	23.97	3.17	10	0.702	0.073	10	39.49	8.80	10			

* (Student's t). # (Welch's t): $P < 0.05$
** (Student's t). ## (Welch's t): $P < 0.01$

Reverse Mutation Test of Arbutin in Bacteria

Reverse Mutation Test of Arbutin in Bacteria

Osamu Yagame, Shinobu Kato and
Toshiaki Kobayashi

1. Introduction

A reverse mutation test of Arbutin using *Salmonella* and *Escherichia coli* was conducted to evaluate its potential for mutagenicity. The assay was conducted according to test methods[2) 3)] in "Guidelines for Toxicity Studies of Drugs Required for the Application for Approval of Manufacture (Import)," as stipulated in the Industrial Safety and Health Law[1)].

The study was conducted from February 9 to 19, 1987.

2. Materials and Methods

2.1 Test and control substances

Arbutin (Lot a, Nippon Fine Chemical Co., Ltd.) was used as the test substance. N-ethyl-N´-nitro-N-nitrosoguanidine, ICR-191 and 2-aminoanthracene were purchased from commercial sources for use as positive control substances. 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) was provided by Professor Matsushima of the Tokyo University Institute of Medical Science.

2.2 Reagents

Agar agar, nutrient broth No. 2, phenobarbital sodium, 5,6-benzoflavone, glucose-6-phosphoric acid, reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced nicotinamide adenine dinucleotide (NADH), L-histidine, D-biotin, L-tryptophan were purchased from commercial sources.

2.3 Test strains

2.3.1 Sources

Salmonella typhimurium TA1535, TA1537 and *Escherichia coli* WP2 *uvr*A (all acquired on May 7, 1974) and *Salmonella typhimurium* TA100 and TA98 (both acquired on December 8, 1975) were obtained from Dr. Tsuneo Kada, Department of Induced Mutation, National Institute of Genetics, were used for the study.

2.3.2 Preservation method

Aliquots (0.8 ml) of bacterial suspensions were mixed with corresponding aliquots (0.07 ml) of dimethylsulfoxide (DMSO) and stored frozen at - 80ºC.

2.4 S9 Mix

2.4.1 S9 source

Source	Date of preparation
Laboratory preparation	September 22, 1986

2.4.2 S9 stock temperature

Stock temperature	- 80ºC

2.4.3 S9 induction method

Animal		Inducer	
Species, strain	Rats, Sprague-Dawley	Name	Phenobarbital sodium (PB-Na) 5,6-benzoflavone (BF)
Sex	Male	Route of administration	Intraperitoneal administration
Age	7 weeks	Administration period and dosage (mg/kg weight)	PB-Na: 30 to 60 mg/kg over 4 days BF: 80 mg/kg, 1 day
Mean body weight	234 g		

2.4.4 S9 Mix composition

Component	Quantity in 1 ml of S9 Mix	Component	Quantity in 1 ml of S9 Mix
S9	0.1 ml	NADH	4 μmol
Magnesium chloride	8 μmol	NADPH	4 μmol
Potassium chloride	33 μmol	Sodium phosphate buffer	100 μmol
Glucose-6-phosphate	5 μmol		

2.5 Adjustment and dosage level of test solution

In the preliminary range-finding test, no toxicity of Arbutin (filtered and sterilized) was observed with any test strain at 5,000 μg/plate. Therefore, the maximum concentration was set at 5,000 μg/plate as one of 6 levels in a common ratio of 2.

Arbutin was dissolved in purified water at a concentration of 50,000 µg/ml. The solution was sterilized by filtration and serially diluted to make 5 additional concentrations of test substance: 25,000, 12,500, 6,250, 3,125 and 1,562.5 µg/ml. Aliquots (0.1 ml) were used for the test.

2.6 Test procedure

The bacterial reverse mutation test was implemented with and without metabolic activation using the preincubation method[4] (at 37ºC for 20 min) in conformity with the "New Guidebook for Mutagenicity Testing in Bacteria" edited by the Chemical Substance Investigation Section, Industrial Safety and Health Department, Labor Standards Bureau, Ministry of Labor, according to the "Guidelines for Toxicity Studies of Drugs Required for the Application for Approval of Manufacture (Import)."[1]

Nutrient broth was inoculated with test strains of bacteria from frozen stocks and cultured overnight (about 10 hours) at 37ºC in a shaking incubator. Aliquots of test substance or solvent were mixed with 0.5 ml of S9 Mix or 0.1 M sodium phosphate buffer solution (pH 7.4). An aliquot (0.1 ml) of culture medium containing either *Salmonella* or *Escherichia coli* was added and the mixture preincubated for 20 minutes at 37ºC. Soft agar was prepared by dissolving agar agar in 0.5%, w/v, sodium chloride solution to a concentration of 0.6%, w/v, and maintained at 45ºC. For use with *Salmonella*, soft agar was mixed at a ratio of 10:1 with an aqueous solution of L-histidine and D-biotin, each at 0.5 mM. For use with *Escherichia coli*, soft agar was mixed at a ratio of 10:1 with an aqueous solution of L-tryptophan at 0.5 mM. Aliquots at double concentration were poured onto minimum glucose agar plates and spread uniformly. Each concentration of test or control substance and the solvent control were assayed in triplicate plates prepared under identical conditions. Plates were incubated for two days at 37ºC and the number of revertant colonies was counted. Growth inhibition was evaluated by

stereomicroscopy.

Solutions of test substance and S9 mix were verified to be aseptic by sterility testing. Solvent negative control and positive control substances with and without S9 mix were evaluated concurrently.

2.7 Criteria for the results

A test substance is evaluated as positive for mutagenicity if the number of revertant colonies is two or more times the number observed in the solvent control plates and if a concentration-dependent increase is evident.

3. Results

Attachment 1 displays the test results.

No increase in the number of revertant colonies was observed with any of the test strains (*Salmonella typhimurium* TA1535, TA1537, TA98, TA100 or *Escherichia coli* WP2 *uvr*A) at any concentration (156.25, 312.5, 625, 1250, 2500 or 5000 μg) irrespective of metabolic activation. The number of revertant colonies was similar to that for the solvent control. Arbutin was nonmutagenic in all test strains under all test conditions.

Positive control substances induced mutagenic responses in respective test strains.

4. Conclusion

Arbutin was nonmutagenic in *Salmonella Typhimurium* TA1535, TA1537, TA98, TA100 and *Escherichia coli* WP2 *uvr*A irrespective of metabolic activation.

References

1) Central Pharmaceutical Affairs Council Recommendation No. 118 (February 15, 1984) Guidelines for Toxicity Studies Required for Applications for Approval to Manufacture (Import) Drugs (Part 1)
2) Ministry of Labor Ordinance No. 261 (May 18, 1986), “Standards for Mutagenicity Testing Using Bacteria”
3) “New Guidebook for Mutagenicity Testing in Bacteria as a Screening Method for Carcinogenicity,” edited by the Chemical Substance Investigation Section, Industrial Safety and Health Department, Labor Standards Bureau, Ministry of Labor, Central Workmen’s Accident Prevention Association (1986).
4) Matsushima, T., Sugimura, T., Nagao, M., Yahagi, T., Shirai, A. and Sawamura, M., In Short-Term Test Systems for Detecting Carcinogens. Proceedings from the Symposium, 1978. Norpoth, K.H. and Garner, R.C., Eds. Springer-Verlag, Berlin, pp. 273-285, (1980)

Attachment-1 Test Results

Name of test substance: Arbutin

Substance	Test substance concentration (μg/plate)	Presence of S9 Mix	Number of reverse mutations (number of colonies/plate)				
			Base pair substitution type			Frameshift type	
			TA100	TA1535	WP2 uvr A	TA98	TA1537
Solvent comparison		–	116 109 (111) 107	14 10 (12) 12	25 22 (25) 27	27 20 (24) 24	11 10 (10) 8
Test substance	156.25	–	128 119 (116) 110	16 14 (14) 11	30 24 (26) 23	31 25 (26) 22	9 11 (10) 11
	312.50	–	115 143 (129) 130	10 10 (10) 10	23 27 (24) 21	22 21 (22) 24	9 9 (10) 11
	625	–	123 115 (120) 121	13 14 (13) 13	23 25 (27) 32	21 21 (22) 23	9 10 (11) 13
	1,250	–	104 106 (106) 108	13 11 (11) 10	32 22 (26) 23	24 31 (27) 25	9 10 (10) 11
	2,500	–	106 112 (112) 117	13 9 (12) 14	23 24 (24) 24	22 20 (23) 28	15 10 (11) 9
	5,000	–	110 116 (114) 115	10 13 (12) 13	34 25 (27) 21	33 26 (27) 22	17 8 (12) 12
Solvent comparison		+	115 139 (132) 143	9 9 (10) 12	19 30 (24) 24	45 39 (40) 37	10 15 (12) 10
Test substance	156.25	+	101 152 (131) 140	10 15 (11) 9	19 31 (25) 25	42 55 (47) 44	10 11 (12) 14
	312.50	+	128 131 (136) 149	14 12 (12) 10	23 20 (25) 31	49 49 (47) 43	12 13 (13) 14
	625	+	102 139 (122) 124	11 14 (13) 13	28 24 (25) 24	47 56 (48) 42	14 14 (13) 12
	1,250	+	106 129 (131) 159	8 9 (10) 13	22 23 (22) 22	42 41 (41) 39	14 14 (12) 9
	2,500	+	159 147 (143) 122	16 14 (15) 14	26 19 (23) 25	42 41 (40) 37	11 10 (11) 12
	5,000	+	123 122 (129) 142	11 10 (11) 11	25 23 (27) 32	43 41 (43) 44	14 16 (14) 12
Positive comparison	Not requiring S9 Mix	Name	* AF-2	N - Ethyl - N´ - Nitro - N - Nitrosoguanidine	N - Ethyl - N´ - Nitro - N - Nitrosoguanidine	* AF-2	ICR-191
		Concentration (μg/plate)	0.01	5	2	0.1	1
		Number of colonies/plate	460 463 (471) 490	911 866 (909) 951	1377 1298 (1283) 1175	541 508 (485) 406	2533 2541 (2601) 2730
	Requiring S9 Mix	Name	2-aminoanthracene	2-aminoanthracene	2-aminoanthracene	2-aminoanthracene	2-aminoanthracene
		Concentration (μg/plate)	1	2	20	0.5	2
		Number of colonies/plate	1081 1324 (1231) 1287	177 128 (143) 125	386 452 (381) 305	332 353 (361) 398	361 319 (348) 364

Remarks: 1) (*) AF-2: 2-(2-furyl) -3-(5-nitro-2-furyl) acrylamide

2) Values represent counts of revertant colonies in individual plates. Values in parentheses represent averages of triplicate plate counts.

Chromosome Aberration Test of Arbutin Using Cultured Mammalian Cells

Chromosome Aberration Test of Arbutin Using Cultured Mammalian Cells

Chiyomi Sugiyama, Hiroshi Kobayashi and
Toshiaki Kobayashi

1. Introduction

Induction of chromosome aberration by arbutin was evaluated using cultured Chinese hamster lung-derived fibroblast cells. The test was conducted according to the mutagenicity testing guidelines (Chromosome Aberration Testing with Mammalian Cells in Culture) in the guidance document regarding toxicity studies required of drugs for the approval of manufacture or import.[1)]

The test was conducted from April 11 to September 5, 1986.

2. Materials and Methods

2.1 Test substance

Arbutin (Lot a, Nippon Fine Chemical Co., Ltd., MW 272.3) was used as the test substance.

2.2 Cells used

2.2.1 Type of cells and passage number

Chinese hamster lung (CHL) fibroblast cells with 25 chromosomes and a doubling time of 15 hours were used. Cells from the 35th passage (CHL-11-35) were used in the cytotoxicity test, from the 25th passage (CHL-11-25) in the chromosome aberration test without exogenous metabolite activation (direct method), and from the 33rd passage (CHL-11-33) in the test with metabolic activation.

2.2.2 Sources

CHL fibroblast cells were obtained from Dr. Hajime Ishidate, Mutagenicity Dept., Safety Center, National Institute of Hygienic Sciences on July 22, 1985.

2.2.3 Preservation method

The cells were cryopreserved (-196ºC, liquid nitrogen) in Eagle's MEM containing 10% dimethyl sulfoxide and 10% calf serum. The cells were subcultured every 3 to 4 days on average in Eagle's MEM containing 10% calf serum.

2.3 Culture solution and reagent

Eagle's MEM containing 10% calf serum (GIBCO)

Serum: GIBCO Lot No. 31N1130 (Direct method)

Lot No. 22P4457 (Metabolic activation method)

0.25% trypsin solution (GIBCO)
Colcemide solution (10 µg/ml, GIBCO)
N-methyl-N´-nitro-N-nitrosoguanidine (Aldrich Chemical Co., Inc.)
Benzo[a]pyrene (Tokyo Kasei)
Oxidized nicotinamide-adenine dinucleotide phosphate (NADP+, Oriental Yeast)
Glucose-6 phosphate (G6P, Sigma)
Phenobarbital sodium (Wako Junyaku)
5,6-Benzoflavone (Aldrich Chemical Co., Inc.)

2.4 S9 Mix

2.4.1 S9 source

Source	Date of preparation
Laboratory preparation	March 18, 1986

2.4.2 S9 storage temperature

Stock temperature	-80ºC

2.4.3 S9 preparation method

Animal		Inducer	
Species, strain	Rats, Sprague-Dawley	Name	Phenobarbital sodium (PB-Na) 5,6 benzoflavone (BF)
Sex	Male	Route of administration	Intraperitoneal administration
Age	7 weeks	Administration period and dosage (mg/kg weight)	PB-Na: 30 to 60 mg/kg over 4 days BF: 80 mg/kg, 1 day
Mean body weight	approx. 262 g		

2.4.4 S9 Mix x 1 ml composition

Component	Content in 1 ml of S9Mix
S9 (Homogenization of liver in 3 volumes (wet weight basis) of 0.15 M KCl)	0.4 ml
$MgCl_2$	5 μmol
KCl	78 μmol
Buffer solution: HEPES (pH 7.2)	4 μmol
$NADP^+$	4 μmol
Glucose-6-phosphate	5 μmol

2.5 Solvent

Physiological saline

2.6 Adjustment of test solution

For the preliminary cytotoxicity test, test solutions were prepared by dissolving the test substance in physiological saline at concentrations of 29.92, 14.96, 7.48, 3.74, 1.87, 0.94 and 0.47 mg/ml. For the chromosome aberration test, the test substance was dissolved in physiological saline at concentrations of 29.92, 14.96, 7.48 and 3.74 mg/ml.

2.7 Dosing level

A preliminary cytotoxicity test was conducted starting at a maximum concentration of 10 mM (2.72 mg/ml culture medium). Growth ratios were as follows: 51% for 2.72 mg/ml, 80% for 1.36 mg/ml, 90% for 0.68 mg/ml and 101% for 0.34 mg/ml (see Attachment 1). Accordingly, the maximum concentration for the chromosome aberration test was established at 2.72 mg/ml and 3 additional concentrations (1.36, 0.68 and 0.34 mg/ml) were established in a common ratio of 2.

2.8 Test procedure

2.8.1 Cytotoxicity test (preliminary test)[2)]

CHL cells on day 4 of cultivation were trypsinized. Cells (1.2×10^4/2 ml) were inoculated on a Petri dish (FALCON) 35 mm in diameter, cultured for 3 days at 37ºC in a 5% carbon dioxide atmosphere and processed for the specimen (test or control substance).

Aliquots (0.2 ml) of the test substance solutions at concentrations described in Section 2.6 were added to duplicate Petri dishes per concentration level and cultured continuously for 2 days. Another 4 Petri dishes were arranged for use as non-processed and solvent comparison controls.

For examination, the culture solution was removed, the dishes were rinsed with physiological saline and fixed for 10 minutes with formalin (10%). The Petri dishes were rinsed in water, and stained with 0.1% crystal violet solution for 10 minutes. Dishes were dried after again rinsing in water.

A monolayer culture density meter (Olympus – Monocellater) was used to measure the cell concentration colorimetrically. The value of the dishes containing non-processed cells represented 100%. On the basis of the measured values, a 50% inhibitory concentration of the test substance was estimated.

2.8.2 Chromosome aberration test (main test)

2.8.2.1 Direct method[2)]

CHL cells (2×10^4/5 ml) were inoculated on Petri dishes (FALCON) 60 mm in diameter, cultured at 37ºC in a 5% carbon dioxide atmosphere and processed for the specimen.

Processing consisted of adding 0.5 ml of the test substance solution to duplicate Petri dishes at concentrations described in Section 2.6. Culture was continued for 24 or 48 hours after processing. Non-processed cells and those processed with ‘solvent only’ represented negative controls. N-methyl-N'-nitro-N-nitrosoganidine was used as the positive control.

2.8.2.2 Metabolic activation method [3)]

CHL cells (2 x 10^4/5 ml) were inoculated on Petri dishes (FALCON) 60 mm in diameter and cultured at 37ºC in a 5% carbon dioxide atmosphere for 3 days.

Culture solution (2.5 ml) was removed from dishes (+S9) assigned to the activated metabolism condition with S9 mix. Culture solution (2 ml) was removed from the dishes (-S9) assigned to the condition without metabolic activation. S9 mix (0.5 ml) was added to +S9 dishes with gentle agitation to assure uniform mixing. Immediately after this, 0.3 ml of the test substance solution at concentrations described in Section 2.6 was added with gentle agitation to each of duplicate dishes per concentration. Test substance solution was similarly added to -S9 dishes in duplicate for each concentration.

After addition of the test solutions, dishes were incubated at 37ºC in 5% carbon dioxide atmosphere for 6 hours. The reaction solution was drained, and 5 ml of Eagle MEM containing 10% calf serum preheated to 37ºC was added for further culturing. Observations were performed 24 hours after processing. Non-processed cells and those processed with 'solvent only' represented negative controls. Benzo[a]pyrene was used as the positive control.

2.8.2.3 Chromosome samples [2)]

Colcemid (final concentration of 0.2 μg/ml) was added 2 hours before sample preparation. The culture medium was transferred to a centrifuge tube. Trypsin (2 ml of 0.25%, 37ºC) was added to the Petri dish to release adherent cells and the dish was incubated at room temperature for 3 to 5 minutes. Cells were aspirated by pipetting, and the solution containing suspended cells was added to the centrifuged tube, stirred, and centrifuged at 1000 rpm for 5 minutes. After aspirating the supernatant, approx. 5 ml of 0.075 M KCI solution was added to the pellet and kept at 37ºC for 15 minutes for hypotonic processing. The culture solution was again centrifuged at 1000 rpm for 5 minutes and the supernatant was discarded. Sedimented cells were then resuspended. Cold fixative (methanol: acetic acid, 3:1) was added gradually, rinsing the wall of the centrifuge tube, and the suspension was stirred. The test solution was again centrifuged at 1000 rpm for 5 minutes, the supernatant discarded and fresh fixative added. This process was repeated 3 times. The final pellet of cells was resuspended with a small quantity of fixative to an appropriate concentration of cells. Suspended cells were applied to a degreased glass slide and air dried.

The specimen was stained in a 1.4% Giemsa solution (pH 6.8, phosphate buffer) for 15 minutes, washed and dried for chromosome examination.

2.9 Observation of chromosome aberrations

Using a 60x to 100x non-cover objective lens, 100 metaphase cells were microscopically examined (600 x to 1000 x) for chromosomal aberrations.

Types of chromosomal structural aberration were classified as shown below. The number of cells with each type of aberration was recorded. Frequency of occurrence of polyploidy was also recorded as the numerical aberration.

Classification of chromosome aberration

Structural aberration:
Gap (including chromatid gap and chromosome gap)
Chromatid break (ctb)
Chromatid exchange (quadriradial, etc.; cte)
Chromosome break (csb)
Chromosome exchange (dicentric, ring, etc.; cse)
Other (fragmentation, etc. , excluding pulverization)
Numerical aberration:
Polyploidy

2.10 Criteria for test results

The rate of chromosome aberrations in CHL cells almost never exceeds 3% for non-processed and solvent-processed cells; therefore, a rate of abnormal cells of less than 5% is declared as (-), 5 to 10% as (±), 10 to 20% as (+), 20 to 50%. as (++), and over 50% as (+++).[2)]

3. Results

Attachments 1 to 3 display the results of the cytotoxicity test (preliminary test) and chromosome aberration test (direct and activated metabolism methods).

In the cytotoxicity test, the growth ratio for arbutin was 51% for 2.72 mg/ml, 80% for 1.36 mg/ml, 90% for 0.68 mg/ml and 101% for 0.34 mg/ml (see Attachment 1). In the chromosome aberration test (direct method), the test substance was not observed to induce aberration of chromosomal structure at concentrations of 2.72, 1.36, 0.68 and 0.34 mg/ml at either 24 or 48 hours of exposure. Polyploidy was not observed. N-methyl-N´-nitro-N-nitrosoganidine used as the positive comparison substance did induce chromosomal structural aberrations. Neither induction of chromosomal structure aberration nor occurrence of polyploidy was observed with physiological saline used as the negative control substance (see Attachment 2). No induction of chronosomal structure aberration was observed in +S9 (metabolic activation) and -S9 dishes at concentration levels of 2.72, 1.36, 0.68 and 0.34 mg/ml. Polyploidy was not observed to occur. Benzo[a]pyrene used for the positive control substance was observed to induce a chromosomal structure aberration under metabolic activation conditions (+S9). Neither induction of

chromosomal structure aberration nor occurrence of polyploidy was observed with physiological saline used as a negative control substance (see Attachment 3).

4. Conclusion

Arbutin did not induce chromosome aberration in fibroblast cells originating from Chinese hamster lung irrespective of metabolic activation.

5. References

1) Central Pharmaceutical Affairs Council Recommendation No. 118 (dated Feb. 15, 1984): “Guidelines for Toxicity Tests Required for Applications for Approval to Manufacture (Import) Drugs” (Part 1)
2) Hajime Ishidate: Detection Method of Mutagen from Chromosome Aberration, Mutagens and Toxicity 4:64-73 (1978).
3) Toshio Sofuni and Atsuko Matsuoka: Metabolic Activation Method in a Chromosome Aberration Test, Environmental Mutagen Research 5 (2):4-6 (1983).

Attachment 1

Cytotoxicity Test of Arbutin (Preliminary test)

	Concentration (mg/ml)	Growth ratio (%)
Growth ratio of cell (Non-processed group = 100%)	2.72	52 49 (51)
	1.36	82 78 (80)
	0.68	86 93 (90)
	0.34	89 113 (101)
	0.17	84 88 (86)
	0.085	109 109 (109)
	0.043	108 112 (110)

Remarks: Values represent individual plates. Values in parentheses represent averages of duplicate plates.

Attachment 2 Chromosome Aberration Test of Arbutin (Direct method)

Substance	Concentration (mg/ml)	Time (h)	Number of cells observed	Occurrence ratio of cells with chromosomal structure aberration (%)									Polyploidy	
				Gap	Chromatid break	Chromatid exchange	Chromosome break	Chromosome exchange	Other	Including total aberration number and gap	Excluding total aberration number and gap	Evaluation	Number of polyploid cells (%)	Evaluation
Non-processed		24	100	0	0	0	0	0	0	0	0	–	0	–
			100	0	0	0	0	0	0	0	0	–	0	–
		48	100	0	0	0	0	0	0	0	0	–	0	–
			100	0	0	0	0	0	0	0	0	–	0	–
Solvent control (physiological saline)	100.00	24	100	0	1	0	0	0	0	1	1	–	0	–
			100	0	0	0	0	0	0	0	0	–	1	–
		48	100	0	1	0	0	0	0	1	1	–	1	–
			100	0	0	0	0	0	0	0	0	–	1	–
Arbutin	0.34	24	100	0	0	0	0	0	0	0	0	–	0	–
			100	0	0	0	0	0	0	0	0	–	0	–
		48	100	0	0	0	0	0	0	0	0	–	0	–
			100	0	0	0	0	0	0	0	0	–	1	–
	0.68	24	100	0	0	0	0	0	0	0	0	–	0	–
			100	0	0	0	0	0	0	0	0	–	1	–
		48	100	0	0	0	0	0	0	0	0	–	1	–
			100	0	0	0	0	0	0	0	0	–	1	–
	1.36	24	100	0	0	1	0	0	0	1	1	–	0	–
			100	0	0	0	0	0	0	0	0	–	1	–
		48	100	1	0	0	0	0	0	1	0	–	0	–
			100	1	0	1	0	0	0	2	1	–	0	–
	2.72	24	100	1	0	0	0	0	0	1	0	–	1	–
			100	1	0	0	1	0	0	2	1	–	0	–
		48	100	0	1	1	0	0	0	2	2	–	0	–
			100	0	0	1	1	0	0	2	2	–	0	–
Positive control MNNG	2.5μg	24	100	3	6	60	0	0	0	65	64	+++	0	–
			100	3	4	53	0	0	0	55	54	+++	1	–
		48	100	4	9	22	1	4	1	34	33	++	5	+ / –
			100	1	8	18	1	2	1	29	28	++	1	–

Remarks: 1) Numerical values represent measured values of the two plates.
2) MNNG: N-methyl-N´-nitro-N-nitrosoguanidine
3) Gap: Including both chromosome gap and chromatid gap; Chromosome exchange: dicentric, ring chromosome, etc.; Other: fragmentation, etc. (excluding pulverization)

Attachment 3 Chromosome Aberration Test of Arbutin (metabolic activation conditions)

Substance	Concentration (mg/ml)	Time (h)	Number of cells observed	Occurrence ratio of cells with chromosomal structure aberration (%)									Polyploid	
				Gap	Chromatid break	Chromatid type exchange	Chromosome break	Chromosome exchange	Other	Including total aberration number and gap	Excluding total aberration number and gap	Evaluation	Number of polyploid cells (%)	Evaluation
Non-processed		+S9	100	0	0	0	0	0	0	0	0	–	0	–
			100	0	0	0	0	0	1	1	1	–	0	–
		-S9	100	0	0	0	0	0	0	0	0	–	0	–
			100	0	0	0	0	0	0	0	0	–	1	–
Solvent control (physiological saline)	100.00	+S9	100	0	0	0	0	0	1	1	1	–	0	–
			100	0	0	0	0	0	0	0	0	–	2	–
		-S9	100	0	0	0	0	0	0	0	0	–	0	–
			100	0	0	0	0	0	0	0	0	–	0	–
Arbutin	0.34	+S9	100	0	0	0	0	0	0	0	0	–	0	–
			100	0	0	0	0	0	0	0	0	–	0	–
		-S9	100	0	0	0	0	0	0	0	0	–	1	–
			100	0	0	0	0	0	0	0	0	–	0	–
	0.68	+S9	100	0	0	0	0	0	0	0	0	–	0	–
			100	0	0	0	0	0	0	0	0	–	0	–
		-S9	100	0	0	0	0	0	0	0	0	–	1	–
			100	0	0	0	0	0	0	0	0	–	0	–
	1.36	+S9	100	0	0	0	0	0	0	0	0	–	2	–
			100	0	0	0	0	0	0	0	0	–	0	–
		-S9	100	0	0	0	0	0	0	0	0	–	1	–
			100	0	0	0	0	0	0	0	0	–	1	–
	2.72	+S9	100	0	0	0	0	0	0	0	0	–	0	–
			100	0	1	0	0	0	0	1	1	–	2	–
		-S9	100	0	1	0	0	0	1	2	2	–	2	–
			100	0	0	0	0	0	0	0	0	–	0	–
Positive comparison B[a]P	40μg	+S9	100	1	11	72	0	0	1	75	75	+++	0	–
			100	0	14	66	2	0	0	74	74	+++	0	–
		-S9	100	1	2	0	0	1	0	3	2	–	1	–
			100	0	0	0	0	0	1	1	1	–	1	–

Remarks: 1) Numerical values represent measured values of the two plates.
2) B[a]P: Benzo[a] pyrene
3) Gap: Including both chromosome gap and chromatid gap; Chromosome exchange: dicentric, ring chromosome, etc.; Other: fragmentation, etc. (excluding pulverization)

Test Report

Percutaneous Carcinogenicity Study of Arbutin in Mice

Project No. H-92070

I. Summary

Percutaneous toxicity and carcinogenicity of Arbutin were evaluated in mice. Groups of 50 Crj:CD-1 (ICR) mice of each sex received Arbutin in 50% ethanol applied to the skin of the back. Doses of 0 (vehicle control group), 45, 135 and 400 mg/kg Arbutin were applied to mice for 78 weeks.

There was no significant difference in mortality between control and dosed groups during the dosing period. There were no clinical signs attributed to exposure to the test substance.

No remarkable changes were noted in body weight and food consumption.

No changes associated with the test substance were observed in any hematology parameter including red blood cell count, white blood cell count and white blood cell differential count.

There were no remarkable differences in absolute or relative organ weights between control and dosed groups. No test substance-related gross lesions were observed at necropsy.

No non-tumor or tumor lesions were observed that were related to the administration of test substance.

In conclusion, the ‘no observed adverse effect level’ of Arbutin was considered to be 400 mg/kg in this study, and there was no evidence of carcinogenic activity of the test substance.

II. Experimental design

1. Objectives

This study evaluated the toxicity and carcinogenicity of Arbutin after application to the dorsal skin of mice for 78 weeks in accordance with a protocol following “Standards concerning the implementation of safety tests on pharmaceuticals” issued by the Ministry of Health and Welfare (*Yakuhatsu* No. 3l3: Mar. 31, 1982), “Partial revision of the standard concerning the implementation of safety tests on pharmaceuticals” (*Yakuhatsu* No. 776: Oct. 1, 1983), “Revision of regulations concerning GLP and inspection of pharmaceuticals” (*Yakuhatsu* No. 870: Oct. 5, 1988), “Guideline on toxicity tests required for applications for approval of manufacture (importation) of drugs” (*Yakushin* 1 No. 24: Sep. 11, 1989), and “Revision of the guideline for single and repeated dose toxicity studies” (*Yakushinyaku* No. 88: Aug. 10, 1993).

2. Sponsor:

Name: Shiseido Safety Research Labs.
Address 1050 Nippa-cho, Kohoku-ku, Yokohama, Kanagawa

3. Contractor

Name Jitsuiken Co., Ltd.
Address: 3303-58 Oaza Odo, Azuma-cho, Azuma-gun, Gunma
(Previous address: 3-13-8 Hacchobori, Chuo-ku, Tokyo)

4. Facility

Name: Haruna Laboratory, Jitsuiken Co., Ltd.
Address: 3303-58 Oaza Odo, Azuma-cho, Azuma-gun, Gunma

Name: Takasaki Pathology Center, Jitsuiken Co., Ltd.
Address: 416 Oaza Nakasatomi, Haruna-machi, Gunma-gun, Gunma

5. Schedule

Approval of protocol:	Mar. 12, 1993
Acquisition of animals:	Mar. 26, 1993
End of quarantine, acclimatization and grouping:	Apr. 7, 1993
First dose:	Apr. 8, 1993
Pathology:	Oct. 11 to 21, 1994
Submission of draft report:	Jul. 15, 1996
Submission of final report:	Sept. 11, 1996

6. Storage of Records, Materials and Specimens

(1) Place

Name: Haruna Laboratory, Jitsuiken Co., Ltd.

Address: 3303-58 Oaza Odo, Azuma-cho, Azuma-gun, Gunma

(2) Period

For ten years after the end of the study. Extension of storage may be negotiated with the sponsor.

(3) Records and specimens

1) Protocol and revised protocol
2) Project Log
3) Materials concerning the test substance (receipt and return invoice)
4) Test substance retains
5) Animal delivery receiving voucher
6) Records of quarantine and acclimatization
7) Records of group assignments
8) Records on the disposition of unused animals
9) Records of the animal room environment
10) Mixing instructions, test substance disposition log and the records of preparation
11) Analytical records for prepared test substance
12) Fur clipping records
13) Dosing records
14) Observation records of clinical signs
15) Records of external palpation for masses
16) Records of body weight
17) Records of food consumption
18) Records of hematology
19) Blood smear specimens
20) Records of organ weights
21) Necropsy findings and photographs
(including negative films of representative cases)
22) Organ and tissue specimens (fixed in 10% neutral buffered formalin)
23) Records on histopathological preparation
24) Tissue blocks
25) Histopathological preparations
26) Histopathological findings and photographs
(including negative films of representative cases)
27) Analytical records for feed and water

28) Qualifications and training records for staff involved in the study
29) Statistical records
30) QA-related documents
31) Copy of the draft report
32) Copy of the final report
33) Records of communications

7. Staff and their assignments

* Planning protocol:	Kenji Suzuki
* Administrative control and management:	Kenji Suzuki, Akira Fukutome
* Reporting:	Akira Fukutome
* Control of test substance:	Kishio Hashizume, Kazuo Hachisuka, Mitsuyuki Katagai, Junei Ichiba, Yukiharu Koike
* Preparation and dosing of test substance:	Kazuo Hachisuka, Mitsuyuki Katagai, Kishio Hashizume, Tomoyasu Takahashi
* Observation of clinical signs:	Kazuo Hachisuka, Mitsuyuki Katagai, Kishio Hashizume, Tomoyasu Takahashi, Taisaburo Hashizume, Saeri Mochizuki, Akira Fukutome, Masaaki Shirai
* Measurement of body weight and food consumption:	Kazuo Hachisuka, Mitsuyuki Katagai, Tomoyasu Takahashi, Kishio Hashizume, Taisaburo Hashizume
* Blood-sampling from caudal vein:	Masaaki Shirai, Yukihisa Karasawa, Kazuo Hachisuka, Mitsuyuki Katagai
* Hematology:	Yasuo Kabe, Noriaki Karasawa, Mutsumi Takano, Shuichiro Maeda, Masao Takano
* Necropsy:	Akira Fukutome, Masaaki Shirai

* Pathological anatomy: Masaaki Shirai, Tomio Yumoto, Junji Koike, Yasuhiro Otsuka, Kazuo Hachisuka, Mitsuyuki Katagai

* Measurement of organ weight: Yukio Tanaka, Masahiro Kasumi

* Preparation of histopathological specimens: Toriko Marushige, Masakazu Yuki, Fumiko Kioka, Manami Hirooka

8. Unexpected situations and deviations from protocol

There were no unexpected situations or deviations from protocol that might have adversely affected the integrity of the study.

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This report was written by:

- Signed - Dated Sep. 11, 1996

III. Materials and Methods

1. Test substance

Shiseido Arbutin (Lot No. TDD-422) provided by Shiseido was used as the test substance. The control groups were given the same dose volume of vehicle (50% ethanol) as dosing groups.

The test substance was a 100% pure, white to grayish-white powder, and almost odorless.

The sponsor determined that the test substance is stable for three (3) years at room temperature.

During the present study, the test substance was stored at room temperature under desiccation, protected from light.

2. Preparation and analysis of dosing solutions

The vehicle was prepared with ethanol (special grade, Wako Junyaku Co., Ltd.) and water for injection, J.P. (Fuso Yakuhin Kogyo Co., Ltd.). The test substance was dissolved in 50% ethanol. Dosing solutions were prepared once every two weeks. The stability of the dosing solutions for at least 2 weeks was verified in preliminary testing[1)]. Aliquots of the dosing solutions were dispensed into amber glass bottles and stored for 12 days at room temperature in the test substance room.

The analysis of dosing solutions was conducted by the sponsor once prior to the study and every three months during the study. Each concentration was found to be within target levels.

3. Animals

Male and female (230 each) Crj:CD-1 (ICR) mice, four-weeks old, were obtained from Charles River Japan Co., Ltd. (795, Shimofurusawa, Atsugi, Kanagawa). Body weights upon arrival were 18.1 to 22.4 g in male and 15.8 to 20.7 g in female mice. Animals were quarantined and acclimatized for 13 days from March 26, 1993 to April 7, 1993. Two hundred male and 200 female mice were selected for the study (body weight on the first dosing day: 26.2 to 33.1 g in male and 19.9 to 26.0 g in female mice).

4. Housing conditions

The animals were individually housed in polycarbonate cages (125W× 200D×110H) with bedding chips (Clean Chip, Nippon Clea Co., Ltd., 2-20-14, Aoba-dai, Meguro-ku, Tokyo).

The animal quarters (Room No. 5, Building No. E) were maintained as follows:

Temperature	:	22 ± 2ºC
Humidity	:	55 ± 10% RH
Ventilation	:	10 to 15 times/hour
Lighting	:	12 hours/day (From 6.00 a.m. to 6.00 p.m., illumination intensity: 150 to 300 lux.)

The room was cleaned daily and disinfected with benzethonium chloride (High Amine Solution, Sankyo Co., Ltd.) solution diluted 1:400. Cages, drinking water bottles, and bedding chips were exchanged twice per week with autoclaved (121ºC, 30 min) replacements. Wire-mesh cage covers were rotated once every five weeks with autoclaved replacements.

Feed and water were available *ad libitum*. CE-2, laboratory chow (for breeding) (Nippon Clea Co., Ltd. 2-20-14, Aoba-dai, Meguro-ku, Tokyo), was provided on the wire-mesh cover, and tap water was replenished three times per week in polycarbonate drinking water bottles (250 mL).

The animals were identified with a tattoo (abbreviated animal number in the caudal area: least significant digit of the group number and the two least significant digits of the individual animal number). Cages were individually identified with a color label (describing the study number, dose, group, test substance, first dosing day, and animal identification number).

Food was analyzed for contaminants by Tokyo Kenbikyo In (Tokyo Institute of Microscopes, 4-8-32, Kudan Minami, Chiyoda-ku, Tokyo).. Analysis of drinking water was performed by Kankyo Giken (Institute of Environmental Technologies, 1709, Kaneko, Gunma-machi, Gunma-gun, Gunma), Environmental Sanitation Test Center, Society of Pharmacists of Gunma Prefecture (5-18-36, Nishi-Katagai-cho, Maebashi, Gunma) and Nakanojo Health Center of Gunma Prefecture (183-1, Nishi-Nakanojo, Nakanojo-machi, Azuma-gun, Gunma). The results were within the allowable limits.

5. Groups

The animals were divided into 4 groups as follows. Each group consisted of 50 male and 50 female mice. Assignment was by stratified randomization based on body weight on the final day of the quarantine and acclimatization period. The table below shows the grouping structure and treatments.

Groups	Dose	Number of animals		Animal No.	
	(mL/kg)	Male	Female	Male	Female
Vehicle control	2	50	50	00M01 to 00M50	00F01 to 00F50
45 mg/kg	2	50	50	01M01 to 01M50	01F01 to 01F50
135 mg/kg	2	50	50	02M01 to 02M50	02F01 to 02F50
400 mg/kg	2	50	50	03M01 to 03M50	03F01 to 03F50
Total		200	200		

6. Rationale for dose and dosing method

No changes attributable to the dosing with the test substance were observed in the 45, 135 and 400 mg/kg groups of a 13-week preliminary toxicity test of Arbutuin[1)] previously conducted. Because of the limited solubility of the test substance and the achievable dose volume, the maximum technically feasible dose is 400 mg/kg. The high dose was therefore 400 mg/kg, the same as in the preliminary study. Middle and low dose levels were 135 mg/kg and 45 mg/kg, calculated with common ratio of 3.

The application site was the interscapular skin (approximately 2×2 cm) clipped with an electrical clipper (edge: 0.5 cm) once every one or two weeks. Dose (2 mL/kg) was calculated according to the most recent body weight. Test substance was applied to the animals using a glass syringe (0.25 mL) daily for 6 days per week (excluding Sundays) for 78 weeks. The route of dosing was chosen to be the same as the clinical route.

7. Observation, measurement and examination

Day 0 was defined to be the first dosing day. The following observations, measurements and examinations were performed on all mice:

(1) Clinical signs

Clinical signs were observed twice per day (before and after dosing). Presence of dead or moribund animals was checked twice per day (morning and afternoon) and moribund sacrifices were conducted when required. In addition to clinical signs, body surfaces were palpated to for masses once per week (Wednesday) from the 26th week of dosing. Documentation of detected masses included date, site, size and progression.

Observations were conducted once per day on Sundays.

(2) Mortality

The date that an animal was found dead or killed in extremis was recorded to calculate mortality.

(3) Body weight

Body weight was measured by an electronic balance (Sartorius Co., Ltd.) once per week (Thursdays) until the 26th week and once every two weeks thereafter.

(4) Food consumption

As for body weights, food consumption was measured by an electronic balance (Sartorius Co., Ltd.) once per week (Fridays) until the 26th week and once every two weeks thereafter.

(5) Hematology

During the 78th week, 0.02 mL of blood was collected from the caudal vein of surviving male and female mice. The blood samples were mixed with 10 mL of diluent (Celpak PK-30L, Toa Iyo Denshi Co.). After mixing, red blood cell count, white blood cell count, and differential count [lymphocyte (Lympho), eosinophil (Eosino), monocyte (Mono), basophil (Baso), band neutrophil (Stab), and segmented neutrophil (Seg)] were measured as indicated in the table below. Hematology was performed whenever possible on moribund animals.

Parameters

Parameters	Method	Equipment
Red blood cell count	Electrical impedance	Multi-item automatic hemocytometer (blood cell counter) M-2000[a)]
White blood cell count	Electrical impedance	Multi-item automatic hemocytometer (blood cell counter) M-2000[a)]
White blood cell ratio	Pappenheim stain	Light microscope, white blood cell classification computer F-410[b)]

a): Toa Iyo Denshi Co., Ltd.

b): Elma Co., Ltd.

(6) Necropsy

All surviving and moribund animals were sacrificed by exsanguination from the abdominal aorta under anesthesia. Body surfaces, intracranial tissues, and internal organs in the thoracic and abdominal cavities were examined. Animals found dead or killed in extremis were immediately subject to necropsy, and observations were recorded as for sacrificed animals. Masses were documented as to site, shape, size and number.

(7) Organ weights

The following organs were weighed at necropsy surviving animals: brain, heart, lung, liver, kidney (right and left), spleen, testis (right and left) and ovary (right and left). Relative weight (weight of organ in g or mg per 100 g of the body weight) was also calculated based on the body weight on the day of necropsy.

(8) Histopathology

Organs and tissues of all animals were fixed in 10% neutral buffered formalin solution (glutaraldehyde formalin fixative for eyes, excluding dead animals). The specimens were embedded in paraffin, and sections were stained with hematoxylin and eosin for microscopy. Animals found dead or killed in extremis during dosing were also examined similarly whenever possible.

There was no difference in incidents of non-tumor and tumor lesions between high dose and control groups. Histopathological examination was therefore performed only on the following organs and tissues of all sacrificed male and female mice from the high dose and control groups in accordance with the protocol: brain, pituitary gland, thyroid gland (including parathyroid when possible), salivary glands (submandibular gland, sublingual gland, right and left), thymus, heart, lung, trachea, bronchus, liver, gallbladder, spleen, pancreas, kidneys (right and left), adrenal glands (right and left), testes (right and left), seminal vesicles (right and left), prostate, ovaries (right and left), uterus, vagina, urinary bladder, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, sternum and femur (including marrow, right or left), spinal cord, eyes (right and left), Harderian glands (right and left), mammary gland (of females in principle), mesenteric lymph nodes, skin at the administration site, skin (other than the administration site) and other organs or tissues with gross lesions (including tumor lesions).

Proliferative lesions were frequently observed in liver, lung and lymphatic tissues. Histopathological definitions for adenocarcinoma, adenoma, nodular hyperplasia and altered cell foci in the liver; adenocarcinoma, adenoma and hyperplasia in the lung; and malignant lymphoma and lymphocytic hyperplasia in the lymphatic tissues are as follows.

<Liver>

Altered cell foci:

Consisting of a few cells and/or a number of lobes. Clear boundary but lack of compression of surrounding tissue. No structural atypia in foci and transition of altered hepatic cords to normal hepatocytes.

Nodular hyperplasia:

Nodular proliferation of hepatocytes with compression of surrounding tissue, unclear lobular structure, but lack of cellular atypia. Accompanied by degeneration and/or necrosis of peripheral hepatocytes, proliferation of oval cells, and bile duct hyperplasia.

Adenoma:

Larger than a lobe and accompanied by cystic degeneration or proliferation of oval cells, with clear boundary and compression. Lack of portal area and disappearance of lobular structure.

Adenocarcinoma:

Accompanied by cellular atypia, local invasion and highly frequent mitotic figures. Cordal, solid or acinar structure without normal sinus.

<Lung>

Hyperplasia:

Focal to diffuse proliferation of normal-appearing cells without compression.

Adenoma:

Cordal distension or papillary proliferation with compression of surrounding tissues and a clean boundary. Lack of atypia and mitotic figure, rare stroma.

Adenocarcinoma:

Various cellular size and proliferation patterns. Atypism, mitotic figures and extensive stroma. Glandular structure rather than papillary or funicular. Invasion into lymphatics, blood vessels, and surrounding parenchyma.

<Lymphatic tissue>

Lymphocytic hyperplasia:

Focal to diffuse proliferation of lymphocytes in follicles or paracortical areas, but with retention of reticulum fiber structure.

Malignant lymphoma:

Nodular to diffuse proliferation of lymphocytes accompanied by distortion of the reticulum fiber structure. Invasion (metastasis) into the capsule, surrounding tissue, thymus, lymphocyte, liver, spleen, lung, etc.

8. Statistical analysis

Body weight, food consumption, hematology, and organ weight data were analyzed first with Bartlett's test for homogeneity of variance. For normal distributions, one-way ANOVA was performed. Pairwise comparisons between dosed and control groups were assessed by Dunnett's test in balanced samples, and by Scheffé's test in unbalanced samples. For skewed distributions, the Kruskal-Wallis H test was used. The significance was assessed by Dunnett's rank test in balanced samples of each group, and by Scheffé's test in unbalanced samples. For the incidences of findings at necropsy and histopathological findings (non-neoplastic and neoplastic changes), Fisher's exact test was used. Mortality differences were assessed by the Chi-square test.

IV. Results

1. Mortality and clinical signs (Figs. 1 and 2, Tables 1, 2, 3, and 4)

In clinical signs, piloerection, anemia, dehydration, erosion, ulcer (cervical and back regions), lacrimination, swelling of eyelids and other symptoms were observed in the male and female animals of all groups including the vehicle control group. In dead or moribund mice, observations included reduced spontaneous motor activity, sedation, hypothermia, gastromegaly and subcutaneous nodule/mass. Since these symptoms were found in the control group with similar frequency and since these are common spontaneous changes in long-term toxicity studies, these clinical signs were not considered to be caused by the test substance.

The following table shows mortality rates (percentage) at the end of the study.

Sex/Dose (mg/kg)	Cont.	45	135	400
Male	6/50 (12%)	10/50 (20%)	17/50 (34%) **	11/50 (22%)
Female	11/50 (22%)	14/50 (28%)	14/50 (28%)	14/50 (28%)

Cont.: Control

** : $P < 0.01$

A significant increase in mortality was observed in males at 135 mg/kg. It was considered not to be related to test substance, because it was not dose-related and no related non-neoplastic or neoplastic lesions at necropsy or histopathological examination were observed.

2. Body weight (Figs. 3 and 4, Tables 5 and 6)

Statistically significant differences were seen in dosed groups as follows:

Dose (mg/kg)	Sex	Dose period	
		Increased weight gain	Decreased weight gain
45	Male	---	---
	Female	---	---
135	Male	---	Week 52
	Female	---	---
400	Male	---	---
	Female	---	---

--- : No statistically significant difference observed.

Reduced body weight gain was observed in females at 135 mg/kg on the 52nd week. This is regarded as an incidental change, since it is not dose related.

3. Food consumption (Figs. 5 and 6, Tables 7 and 8)

Statistically significant differences were observed in dosed groups as follows:

Dose (mg/kg)	Sex	Dose period	
		Increase	Decrease
45	Male	---	---
	Female	---	Week 28
135	Male	---	Week 21
	Female	---	---
400	Male	Weeks 15, 30, 32	---
	Female	---	Week 28

--- : No statistically significant difference observed.

Since there were no corresponding changes in body weight, these are not regarded as toxicologically significant.

4. Hematology (Tables 9 and 10)

There were no significant changes attributed to the test substance in either sex in any dosed groups in the 78th week.

5. Organ weights (Tables 11 and 12)

The following table indicates organs in which statistically significant differences were observed.

Sex		Male			Female		
Dose (mg/kg)		45	135	400	45	135	400
Organ	Body weight (g)	45.55	45.22	46.31	37.73	37.58	37.45
Spleen	Absolute weight	---	---	---	---	---	↓ 77.4
	Relative weight	---	---	---	---	---	---

↓ : $P < 0.05$

--- : No statistically significant difference observed.

$$\text{Numeric value in the table (\%)} = \frac{\text{Absolute weight of spleen in the 400 mg/kg group}}{\text{Absolute weight of spleen in vehicle group} \times 100}$$

No significant difference related to test substance was observed.

A significant decrease of the absolute weight of spleen in 400 mg/kg females was observed in the 78th week. An increased incidence (compared to control) of splenic hypertrophy was observed in females at 400 mg/kg. This finding was related to proliferative changes (extramedullary hematopoiesis, metastasis of malignant lymphoma, etc.), but is not considered treatment-related.

6. Necropsy (Tables 13 and 14)

There were no remarkable findings related to test substance.

(1) Dead and moribund animals (Week 0 to 52)

Spontaneous lesions were seen rarely in dead or moribund animals in any group, including vehicle control, between Weeks 0 and 52.

(2) Dead and moribund animals (Week 53 to 78)

Major findings in dead and moribund animals from the 53rd to 78th week are shown below. Incidences of these findings were not significantly different from vehicle control group and were not dose-related. Since, these changes are frequently observed in normal mice in long-term toxicity studies, they are considered spontaneous lesions.

Test period	Sex		Male				Female			
	Dose (mg/kg)		Cont.	45	135	400	Cont.	45	135	400
Dead and moribund animals <Week 53 to 78>	Organ	Findings (Number of animals examined)	4	7	12	7	10	13	15	10
	Liver	Nodules/Masses	0	1	3	3	0	1	1	1
	Glandular stomach	Mucosal thickening	0	0	0	1	1	2	1	0
	Lung	Dark red	0	2	3	2	3	1	1	1
		Nodules/Masses	2	1	0	0	2	1	2	0
	Kidney	Discoloration	0	0	0	1	1	3	2	3
		Granular surface	0	0	0	0	1	3	3	1
	Spleen	Hypertrophy	0	3	5	3	8	8	9	6
		Nodules/Masses	0	0	0	0	0	2	0	0
	Thymus	Atrophy	0	1	3	2	1	2	4	2
	Seminal vesicle	Hypertrophy	4	5	9	5	/	/	/	/
	Ovary	Cyst	/	/	/	/	7	9	10	8
	Uterus		/	/	/	/				
		Swelling					6	3	3	4
		Mucosal thickening					1	3	3	1
	Harderian gland	Dark brown	0	0	0	0	2	2	2	1
	Preputial glands			(4)	(7)	(4)	/	/	/	/
		Cyst	0	4	7	4				
	Other lymph nodes	Hypertrophy	0	1	3	1	5	5	2	3
	Urogenital						/	/	/	/
		Nodules	3	2	2	2				

Cont. : Control / : Not examined ( ) : Number of animals examined

(3) Dead and moribund animals (Week 0 to 78)

The major findings are summarized as follows:

Test period	Sex		Male				Female			
	Dose (mg/kg)		Cont.	45	135	400	Cont.	45	135	400
Dead and moribund animals <Week 0 to 78>	Organ	Findings (Number of animals examined)	6	10	17	12	11	16	19	15
	Liver	Nodules/Masses	0	1	3	3	0	1	1	1
	Glandular stomach	Mucosal thickening	0	0	0	1	1	2	1	0
	Lung	Dark red	0	3	3	4	3	1	3	2
		Nodules/Masses	2	1	0	0	2	2	2	0
	Kidney	Discoloration	1	0	0	1	1	3	3	3
		Granular surface	1	0	0	0	1	3	5	2
	Spleen	Hypertrophy	1	5	8	6	9	10	10	9
		Nodules/Masses	0	0	0	0	0	2	0	0
	Thymus	Atrophy	1	1	5	2	1	2	4	2
		Hypertrophy	0	1	0	0	0	2	2	2
	Seminal vesicle	Hypertrophy	4	5	9	5	/	/	/	/
	Ovary	Cyst	/	/	/	/	8	10	10	9
	Uterus		/	/	/	/				
		Swelling					6	4	4	5
		Mucosal thickening					1	3	4	1
	Harderian gland	Dark brown	0	0	0	0	2	2	2	1
	Preputial glands			(5)	(7)	(5)	/	/	/	/
		Cyst	0	5	7	5				
	Other lymph nodes		(1)	(1)	(3)	(4)	(5)	(5)	(2)	(4)
		Hypertrophy	1	1	3	4	5	5	2*	4
	Urogenital						/	/	/	/
		Nodules	3	2	3	4				

Cont. : Control / : Not examined ( ) : Number of animals examined

* : P < 0.05

(4) Surviving animals (Week 78)

Major findings are shown below:

Test period	Sex		Male				Female			
	Dose (mg/kg)		Cont.	45	135	400	Cont.	45	135	400
Surviving animals <Week 78>	Organ	Findings (Number of animals examined)	44	40	33	38	39	34	31	35
	Liver	Granular surface	0	0	0	2	0	0	0	0
		Discoloration	0	0	0	3	0	0	1	0
		Nodules/Masses	14	16	11	10	2	1	1	0
	Glandular stomach	Mucosal thickening	2	0	3	2	5	2	2	3
	Lung	Nodules/Masses	8	9	6	9	5	5	5	9
	Spleen	Hypertrophy	10	6	3	4	14	9	7	6
	Thymus	Atrophy	21	24	19	18	7	4	5	3
		Hypertrophy	2	1	0	0	4	8	1	5
	Testis						/	/	/	/
		Atrophy	2	0	1	0				
		Fragile	1	0	0	1				
		Discoloration	3	3	3	2				
	Seminal vesicle						/	/	/	/
		Hypertrophy	12	10	6	6				
		Discoloration	1	3	0	0				
	Ovary		/	/	/	/				
		Cyst					26	29	25	18
	Uterus		/	/	/	/				
		Swelling					36	31	26	33
	Vagina		/	/	/	/				
		Mucosal thickening					5	0*	0*	0*
	Preputial glands		(41)	(37)	(32)	(37)	/	/	/	/
		Cyst	41	39	32	37				

Cont. : Control / : Not examined ( ) : Number of animals examined

* : P < 0.05

7. Histopathology (Tables 15, 16, 17 and 18)

Non-tumor lesions

There were no treatment-related significant findings.

Significant findings are shown below. Since the incidence of these findings did not show an increasing dose-relationship, and since they are frequently observed spontaneously in long-term toxicity studies, they are regarded as spontaneous lesions.

(1) Dead and moribund animals (Week 0 to 52)

The following table summarizes significant findings and findings that were observed with comparatively high frequency:

Test period	Sex		Male				Female			
	Dose (mg/kg)		Cont.	45	135	400	Cont.	45	135	400
Dead and moribund animals <Week 0 to 52>	Organ	Findings (Number of animals examined)	2	3	5	5	1	3	4	5
	Pituitary gland	Hyperplasia of glandular cells	2	1	0*	(4) 0	0	0	0	1

Cont. : Control / : Not examined ( ) : Number of animals examined

* : P < 0.05

(2) Dead and moribund animals (Week 53 to 78)

The following table summarizes significant findings only.

Test period	Sex		Male				Female			
	Dose (mg/kg)		Cont.	45	135	400	Cont.	45	135	400
Dead and moribund animals <Week 53 to 78>	Organ	Findings (Number of animals examined)	4	7	12	7	10	13	15	10
	Submandibular gland	Monocytic infiltration	2	4	7	3	4	(12) 0*	0*	3
	Trachea	Acidophilic (crystalline) substance in submucosal gland	1	4	6	6	1	4	8*	2
	Sternal bone marrow	Fibrosis	0	0	0	0	1	2	9*	4
	Femoral bone Marrow	Fibrosis	0	0	1	0	0	2	7*	3

Cont. : Control / : Not examined ( ) : Number of animals examined

* : P < 0.05

(3) Dead and moribund animals (Week 0 to 78)

The following table shows significant findings, those with a high incidence and those regarded the cause of death.

Test period	Sex		Male				Female			
	Dose (mg/kg)		Cont.	45	135	400	Cont.	45	135	400
Dead and moribund animals <Week 0 to 78>	Organ	Findings (Number of animals examined)	6	10	17	12	11	16	19	15
	Liver	Leukemia and metastasis of malignant lymphoma	0	1	3	3	2	3	2	4
	Proventriculus	Hyperkeratosis	2	8	15*	9	6	10	11	11
	Glandular stomach	Hyperplasia of mucosal epithelium	1	4	8	2	5	3	6	8
	Heart	Myocardial degeneration and necrosis	1	2	6	3	3	3	2	2
	Lung	Leukemia and metastasis of malignant lymphoma	0	2	2	4	3	3	2	5
	Trachea	Acidophilic (crystalline) substance in submucosal glands	2	4	9	7	1	5	9*	2
	Kidney	Glomerular nephropathy	1	0	3	0	1	3	6	3
		Hypertrophy of renal epithelium	1	5	6	3	1	8*	7	3
		Leukemia and metastasis of malignant lymphoma	0	1	2	4	2	3	4	6
		Metastasis of histiocytic sarcoma	0	0	0	0	4	2	1*	1
	Spleen	Excessive extramedullary hematopoiesis	4	5	8	7	5	8	10	9
		Leukemia and metastasis of malignant lymphoma	0	3	2	4	2	2	3	5
	Sternal bone marrow	Fibrosis	0	0	0	0	1	2	11*	5
	Femoral bone marrow	Fibrosis	0	0	1	0	0	2	8*	4
	Brain	Calcification in thalamus	0	3	6	0	0	0	1	2
	Adrenal gland	Subcapsular hyperplasia	2	5	5	2	9	13	14	9
	Ovary	Cyst	/	/	/	/	11	12	17	11
	Uterus	Cystic endometrial hyperplasia	/	/	/	/	11	13	17	15
	Sternum	Detachment of articular chondrocytes	1	6	13*	5	7	7	12	10
		Increase of trabeculae in bone marrow	2	8	15*	10	8	11	15	13
	Femur	Detachment of articular chondrocytes	3	4	9	8	4	11	12	7
		Increase of trabeculae in bone marrow	2	7	14*	10	9	13	15	11
		Hypertrophy of articular chondrocytes	2	4	9	10	4	10	11	7
	Bulbourethral gland		(2)	(1)	(2)	(4)	/	/	/	/
		Proliferation of glandular cells	2	0	2	3				
		Congestion	2	0	2	2				
		Inflammation	0	1	0	0				

Cont. : Control / : Not examined ( ) : Number of animals examined

* : $P < 0.05$

(4) Surviving animals (Week 78)

The following indicate significant findings and findings observed with comparatively high frequency.

Test period	Sex		Male		Female	
	Dose (mg/kg)		Cont.	400	Cont.	400
Surviving animals <Week 78>	Organ	Findings (Number of animals examined)	44	38	39	35
	Esophagus	Monocytic infiltration	0	4*	1	2
	Glandular stomach	Hyperplasia of mucosal epithelium	36	24*	27	19
	Heart	Myocardial degeneration and necrosis	21	17	5	2
	Spleen			(37)		
		Excessive extramedullary hematopoiesis	26	25	33	33
	Brain	Calcification in thalamus	26	17	6	7
	Adrenal gland	Subcapsular hyperplasia	21	23	37	35
	Seminal vesicle	Cystic glandular tissue	27	15*	/	/
	Ovary	Cyst	/	/	26	22
	Uterus	Cystic endometrial hyperplasia	/	/	37	35
	Thymus	Detachment of articular chondrocytes	40	36	28	26
	Femur	Detachment of articular chondrocytes	26	24	21	18
		Hypertrophy of chondrocytes	23	20	14	16

Cont. : Control / : Not examined

* : $P < 0.05$

Neoplastic lesions

There were no treatment-related findings.

Tumor findings are summarized in the table below. There was no significant difference in tumor frequency from the vehicle control group. Tumor incidence did not show a dose-relationship, and those types of tumors that were found are frequently observed in mice during long-term toxicity studies; thus, these are regarded as spontaneous lesions.

(1) Dead and moribund animals (Week 0 to 52)

The following table shows all neoplastic lesions observed.

Test period	Sex		Male				Female			
	Dose (mg/kg)		Cont.	45	135	400	Cont.	45	135	400
Dead and moribund animals <Week 0 to 52>	Organ	Findings (Number of animals examined)	2	3	5	5	1	3	4	5
	Glandular stomach	(M) Hemangiosacroma	0	0	0	0	1	0	0	0
	Lung	(M) Bronchiolar/ Alveolar adenocarcinoma	0	0	0	0	0	1	0	0
	Thymus		(1)	(2)	(3)					
		(M) Malignant lymphoma	0	0	1	1	0	2	1	3
	Sternal bone marrow	(M) Myelocytic leukemia	0	1	0	0	1	0	0	0
	Femoral bone marrow	(M) Myelocytic leukemia	0	1	0	0	1	0	0	1
	Mesenteric lymph nodes	(M) Malignant lymphoma	0	0	0	1	0	0	0	0
	Other: skin		(1)	(1)	(2)	(1)	/	/	/	/
		(M) Osteogenic sarcoma	0	0	1	0				

Cont. : Control (M) : Malignant tumor / : Not examined ( ) : Number of animals examined

(2) Dead and moribund animals (Week 53 to 78)

The following table summarizes all neoplastic lesions observed.

Test period	Sex		Male				Female			
	Dose (mg/kg)		Cont.	45	135	400	Cont.	45	135	400
Dead and moribund animals <Week 53 to 78>	Organ	Findings (Number of animals examined)	4	7	12	7	10	13	15	10
	Liver	(B) Hepatocellular adenoma	0	1	0	1	0	0	0	0
		(B) Hemangioendothelioma	0	0	0	1	0	0	0	0
		(M) Histiocytic sarcoma	0	0	0	0	1	1	1	1
	Gallbladder					(6)		(12)		
		(B) Papilloma	0	0	1	0	0	0	0	0
	Glandular stomach	(B) Fibroma	0	0	0	0	0	1	0	0
	Lung	(B) Bronchiolar/ Alveolar adenoma	1	0	1	0	2	0	1	0
		(M) Bronchiolar/ Alveolar adenocarcinoma	1	1	0	0	0	0	1	0
	Urinary bladder	(M) Transitional cell carcinoma	0	0	1	0	0	0	0	0
	Spleen	(M) Malignant lymphoma	0	0	0	0	1	0	0	0
	Thymus			(4)		(6)	(8)	(11)		
		(M) Malignant lymphoma	0	1	0	0	1	0	2	1
	Sternal bone marrow	(M) Myelocytic leukemia	0	0	0	1	0	0	0	0
	Femoral bone marrow	(M) Myelocytic leukemia	0	0	0	1	0	0	0	0
	Mesenteric lymph						(9)			
	nodes	(M) Malignant lymphoma	1	1	1	1	2	3	2	2
		(M) Histiocytic sarcoma	0	0	0	0	1	1	0	1
	Testis	(B) Papillary adenoma	1	0	0	0	/	/	/	/
	Uterus	(B) Hemangioendothelioma	/	/	/	/	0	0	0	1
	Mammary gland		/	/	/	/	(9)	(12)		(9)
		(M) Squamous cell carcinoma					0	0	1	0
		(M) Adenocarcinoma					0	1	0	0
	Femur	(M) Osteosacroma	0	0	1	0	0	0	0	0
	Harderian gland	(B) Adenoma	0	0	1	0	0	1	0	0
	Other skin		/	(1)	(1)	/	(1)	(1)	(1)	/
		(M) Basal cell carcinoma		0	0		1	0	0	
		(M) Adenocarcinoma		0	0		0	1	0	
		(M) Osteogenic sarcoma		0	0		0	0	1	
	Other lymph nodes		/	/	(3)	(1)	/	/	/	/
		(M) Malignant lymphoma			1	0				

Cont. : Control (B) : Benign tumor (M) : Malignant tumor / : Not examined ( ) : Number of animals examined

(3) Dead and moribund animals (Week 0 to 78)

The following table shows the total neoplastic lesions observed.

Test period	Sex		Male				Female			
	Dose (mg/kg)		Cont.	45	135	400	Cont.	45	135	400
Dead and moribund animals <Week 0 to 78>	Organ	Findings (Number of animals examined)	6	10	17	12	11	16	19	15
	Liver	(B) Hepatocellular adenoma	0	1	0	1	0	0	0	0
		(B) Hemangioendothelioma	0	0	0	1	0	0	0	0
		(M) Histiocytic sarcoma	0	0	0	0	1	1	1	1
	Gallbladder			(19)		(11)		(15)		
		(B) Papilloma	0	0	1	0	0	0	0	0
	Glandular stomach	(B) Fibroma	0	0	0	0	0	1	0	0
		(M) Hemangiosacroma	0	0	0	0	1	0	0	0
	Lung	(B) Bronchiolar/ Alveolar adenoma	1	0	1	0	2	0	1	0
		(M) Bronchiolar/ Alveolar adenocarcinoma	1	1	0	0	0	1	1	0
	Urinary bladder					(11)				
		(M) Transitional cell carcinoma	0	0	1	0	0	0	0	0
	Spleen	(M) Malignant lymphoma	0	0	0	0	1	0	0	0
	Thymus		(5)	(6)	(15)	(11)	(9)	(13)		
		(M) Malignant lymphoma	0	1	1	1	1	2	3	4
	Sternal bone marrow	(M) Myelocytic leukemia	0	1	0	1	1	0	0	0
	Femoral bone marrow	(M) Myelocytic leukemia	0	1	0	1	1	0	0	1
	Mesenteric lymph nodes						(10)		(18)	(14)
		(M) Malignant lymphoma	1	1	1	2	2	3	2	2
		(M) Histiocytic sarcoma	0	0	0	0	1	1	0	1
	Testis	(B) Papillary adenocarcinoma	1	0	0	0	/	/	/	/
	Uterus	(B) Hemangioendothelioma	/	/	/	/	0	0	0	1
	Mammary gland		/	/	/	/	(10)	(15)		(13)
		(M) Squamous cell carcinoma					0	0	1	0
		(M) Adenocarcinoma					0	1	0	0

Cont. : Control (B) : Benign tumor (M) : Malignant tumor / : Not examined ( ) : Number of animals examined

(3) Dead and moribund animals (Week 0 to 78)(Continued)

Test period	Sex		Male				Female			
	Dose (mg/kg)		Cont.	45	135	400	Cont.	45	135	400
Dead and moribund animals <Week 0 to 78>	Organ	Findings (Number of animals examined)	6	10	17	12	11	16	19	15
	Femur	(M) Osteosacroma	0	0	1	0	0	0	0	0
	Harderian gland									(14)
		(B) Adenocarcinoma	0	0	1	0	0	1	0	0
	Other skin		(1)	/	(4)	(4)	(1)	(1)	(1)	/
		(M) Basal cell carcinoma	0		0	0	1	0	0	
		(M) Adenocarcinoma	0		0	0	0	1	0	
		(M) Osteogenic sarcoma	0		1	0	0	0	1	
	Other lymph nodes		/	/	(3)	(1)	/	/	/	/
		(M) Malignant lymphoma			1	0				

Cont. : Control (B) : Benign tumor (M) : Malignant tumor / : Not examined ( ) : Number of animals examined

(4) Surviving animals (Week 78)

The following table shows lesions observed.

Test period	Sex		Male		Female	
	Dose (mg/kg)		Cont.	400	Cont.	400
Surviving animals <Week 78>	Organ	Findings (Number of animals examined)	44	38	39	35
	Liver	(B) Hepatocellular adenoma	14	13	0	0
		(B) Hemangioendothelioma	1	0	0	0
	Pancreas	(B) Islet cell adenoma	0	0	1	0
	Duodenum	(B) Adenoma	0	0	0	1
	Jejunum	(M) Hemangiosacroma	0	1	0	0
	Rectum	(M) Adenocarcinoma	0	0	0	1
	Lung	(B) Bronchiolar/ Alveolar adenoma	3	7	1	4
		(M) Bronchiolar/ Alveolar Adenocarcinoma	5	3	3	5
	Urinary bladder	(M) Transitional cell carcinoma	1	0	0	0
	Spleen			(37)		
		(B) Hemangioendothelioma	0	0	1	0
		(M) Hemangiosacroma	1	0	0	1

Cont. : Control (B) : Benign tumor (M) : Malignant tumor ( ) : Number of animals examined

(4) Surviving animals (Week 78) (Continued)

Test period	Sex		Male		Female	
	Dose (mg/kg)		Cont.	400	Cont.	400
Surviving animals <Week 78>	Organ	Findings (Number of animals examined)	44	38	39	35
	Thymus			(37)	(36)	(33)
		(M) Malignant lymphoma	2	0	3	2
	Sternal bone marrow	(M) Myelocytic leukemia	0	0	1	0
		(M) Mastocytoma	0	1	0	0
	Femoral bone marrow	(B) Hemangioendothelioma	0	1	0	0
		(M) Myelocytic leukemia	0	0	1	0
		(M) Mastocytoma	0	1	0	0
	Mesenteric lymph nodes		(42)			
		(M) Malignant lymphoma	0	0	1	0
	Pituitary gland	(B) Adenoma	0	0	1	1
	Testis	(B) Interstitial cell tumor	0	1	/	/
	Ovary		/	/		
		(B) Papillary cystic adenoma			1	2
		(M) Undifferentiated gonadotropic tumor			1	0
	Uterus		/	/		
		(B) Leiomyoma			1	2
		(B) Fibroma			0	1
		(M) Leiomyosacroma			2	0
	Harderian gland	(B) Adenoma	5	2	1	2
	Other skin		/	/	(3)	(1)
		(M) Basal cell carcinoma			2	0
	Skeletal muscle (thigh muscle)		/	/	(1)	/
		(M) Rhabdomyosacroma			1	

Cont. : Control (B) : Benign tumor (M) : Malignant tumor

/ : Not examined ( ) : Number of animals examined

(1) Dead, moribund, and surviving animals (Male)

Name of test substance		Arbutuin													
		#				#				#				##	
Test period		< - - -	0 to 52-week		- - - >	< - - -	53 to 78-week		- - - >	< - - -	0 to 78-week		- - - >	< 78-week >	
Dose (mg/kg)		Cont.	45	135	400	Cont.	45	135	400	Cont.	45	135	400	Cont.	400
Number of test animals		2	3	5	5	4	7	12	7	6	10	17	12	44	38
Number of tumors	Benign	0	0	0	0	2	1	3	2	2	1	3	2	23	24
	Malignant	0	1	2	2	2	3	4	2	2	4	6	4	8	5
Total number of tumors		0	1	2	2	4	4	7	4	4	5	9	6	31	29
Number of animals with tumor	Benign	0	0	0	0	1	1	3	2	1	1	3	2	22	20
	Malignant	0	1	2	2	2	3	4	2	2	4	6	4	11	4
Total number of animals with tumor		0	1	2	2	3	4	7	4	3	5	9	6	33	24

#: Dead and moribund animals ##: Surviving animals Cont.: Control

(2) Dead, moribund, and surviving animals (Female)

Name of test substance		Arbutin													
		#				#				#				##	
Test period		< - - -	0 to 52-week		- - - >	< - - -	53 to 78-week		- - - >	< - - -	0 to 78-week		- - - >	< 78-week >	
Dose (mg/kg)		Cont.	45	135	400	Cont.	45	135	400	Cont.	45	135	400	Cont.	400
Number of test animals		1	3	4	5	10	13	15	10	11	16	19	15	39	35
Number of tumors	Benign	0	0	0	0	2	2	1	1	2	2	1	1	6	13
	Malignant	2	3	1	4	7	7	8	5	9	10	9	9	14	9
Total number of tumors		2	3	1	4	9	9	9	6	11	12	10	10	20	22
Number of animals with tumor	Benign	0	0	0	0	2	2	1	1	2	2	1	1	6	11
	Malignant	1	3	1	2	7	6	6	4	8	9	7	6	10	9
Total number of animals with tumor		1	3	1	2	9	8	7	5	10	11	8	7	16	20

#: Dead and moribund animals ##: Surviving animals Cont.: Control

V. Discussion and Conclusion

Toxicity and carcinogenicity of Arbutin were evaluated by applying 0 (vehicle), 45, 135 and 400 mg/kg to the interscapular skin of Crj:CD-1 (ICR) mice for 78 weeks. Each group consisted of 50 male and female mice.

No significant difference was observed in mortality rates between the vehicle control group and dosed groups during dosing. There was no treatment-related difference in clinical signs, body weight or food consumption.

There was no treatment-related difference in red blood cell count, white blood cell count or the white blood cell differential count.

There were no remarkable differences in absolute and relative weights between the vehicle control group and dosed groups.

There were no treatment-related findings at necropsy.

There were no treatment-related findings in microscopic examination. Non-tumor lesions included hyperplasia of mucosal epithelium in the glandular stomach, myocardial degeneration and necrosis in the heart, excessive extramedullary hematopoiesis in the spleen, calcification in thalamus in the brain, subcapsular hyperplasia in the adrenal gland, cyst in the ovary, cystic endometrial hyperplasia in the uterus, and detachment or hypertrophy of articular chondrocytes in the joint. Tumor lesions included hepatocellular adenoma, bronchiolar/alveolar adenoma, and malignant lymphoma. These lesions are frequently observed in aging mice [3, 4, 5)]. Major findings in animals found dead or killed in extremis include disturbances of urogenital system (e.g., inflammation in bulbourethral gland), glomerular nephropathy and malignant lymphoma. These abnormalities may be related to the cause of death [2, 4, 5)].

In consideration of the findings above, the no observed adverse effect level of Arbutin in the present study is estimated to be 400 mg/kg in both male and female mice, and it is concluded that the test substance is not carcinogenic under the conditions of the present study.

VI. References

1) Kenji Suzuki et al.: Preliminary percutaneous carcinogenicity study of Arbutin in mice. (1993), unpublished.
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5) Maita, K., et al.: Mortality, major cause of moribundity, and spontaneous tumors in CD-1 mice. Toxicol. Pathol. 16:340-349 (1988).

Reproduction study of Arbutin in Rats by Subcutaneous administration

CONTENTS

Introduction

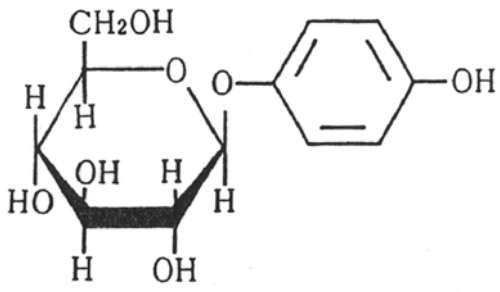
To evaluate the effects on reproductive parameters in rats, Arbutin was injected subcutaneously into female rats for a period from before pregnancy to the end of lactation and into male rats for a period from 9 weeks prior to mating to the completion of mating.

Materials and Methods

1. Test Substance

Arbutin was provided by the Shiseido Research Institute. The name and code number of the test substance are as follows:

Code No. : SL-16
Lot No. : a
General name : Arbutin
Chemical substance name : Hydroquinone-β-D-glucopyranoside
Structural formula :



Molecular formula : $C_{12}H_{16}O_7$
Molecular weight : 272.25
Solubility : In water, 7% by weight at 0ºC, 16% by weight at 25ºC, 37% by weight at 50ºC, and in a hydroalcoholic solution (1:1), 13% by weight at 0ºC, 25% by weight at 25ºC, 32% by weight at 37ºC. Readily soluble in DMSO.
Storage method : The test substance was stored in a cold room designated for reagents and specimens (approx. 4ºC, Locker No. L-31). Dosing solutions of test substance were shielded from the light and stored in a box-type plastic container in a cold room (Locker No. L-32) or in a refrigerator in the animal facility.

A sample of the test substance was retained at the test facility and the remainder was returned to the sponsor after completion of

the study.

2. Animals

2.1 Species

Crj: CD rats (SPF) were used. The SD strain is commonly used in this type of toxicity study and was preferred by the sponsor. Rats have been used frequently in this laboratory.

2.2 Purchases and Acclimatization

Males and females (155 each) were purchased from Charles River Japan (795, Furusawa, Atsugi, Kanagawa). Male rats were 5 weeks of age and purchased on March 10, 1986. Female rats were 7 weeks of age when purchased on April 28, 1986. Body weight range was 116 to 142 g for males and 142 to 173 g for females upon arrival. During the one-week acclimatization period, both males and females were housed in groups of 5 in each suspended wire-mesh cage (maximum length: 24 × 38 × 20 cm).

2.3 Feed

Throughout the test period, rats were housed individually in the wire mesh cages described above. Female rats were housed in plastic cages (28 × 43 × 20 cm) from mid-pregnancy to the end of lactation. Animals were fed laboratory chow for breeding (NMF, Oriental Yeast Industry) and tap water *ad libitum*.

Animal quarters were maintained at 23 ± 1ºC and 53 ± 7% relative humidity, under a 12-hour light-dark cycle (on from 7:00 to 19:00).

Analysis of feed and drinking water, and review of temperature and humidity records did not reveal any conditions expected to have had an adverse effect on the study results.

2.4 Animal identification

The rats were identified with individual numbers marked on their tails with indelible ink. Cages were labeled with color-coded vinyl tape bearing individual animals numbers of the group.

3. Grouping

At the end of acclimatization, before the first dosing, both male and female rats were grouped. Rats (140 females and males each) were divided into 4 groups. Each group consisted of 35 rats per sex. Assignment of rats to treatment groups was by stratified randomization on the basis of weight. Randomization was by a table of random numbers.

4. Preparation of Test Samples

Test substance was dissolved in physiological saline. Concentrations were varied in order to maintain a constant dosing volume of 2 ml/kg body weight. Glassware used in preparation of dosing solutions was sterilized by boiling. The measuring flask was sanitized with 70% alcohol before use.

Dosing solutions of test substance were prepared once per week. The test substance was determined to be stable in saline for two weeks. The concentration of test substance in dosing solutions was analyzed by the sponsor once every three preparations. Each concentration was verified in six such analyses.

5. Dosing

5.1 Dosage

The highest dose level was 400 mg/kg, which is limited by the test substance's solubility (20% w/v solution) at the dosing volume of 2 ml/kg. The middle dose level was 100 mg/kg and the low dose level was 25 mg/kg. Another group dosed with vehicle (physiologic saline) was provided for control.

5.2 Route and dosing volume

The subcutaneous route was chosen as it approximates the clinical route of administration (dermal). Dosing solutions were injected subcutaneously in the back of the rats once per day between 9:00 and 12:00 in the morning.

Dosing volume for the male rats was calculated on the basis of body weight, measured twice per week. For female rats, dosing volume was calculated according to the most recently measured body weight.

5.3 Dosing duration

Test substance or vehicle was administered to male rats for 9 weeks prior to mating, from 6 to 15 weeks of age. Dosing continued until the rats were observed to have successfully copulated.

For female rats, dosing was for two weeks before mating, i.e., from 8 to 10 weeks of age. Dosing continued during the mating period. Of the female rats observed to have successfully copulated, the group of approximately 20 that were scheduled for caesarean section was dosed every day from Days 0 to 19 of pregnancy. They were sacrificed on Day 20 of pregnancy. The remaining animals that were scheduled to go to term (approximately 10) were dosed daily from Day 0 of pregnancy to Day 21after parturition.

5.4 Group composition

The following animals were allocated to each dosage group and assigned individual numbers:

Group	Dosage (mg/kg/day)	Number of animals and individual number	
		Male	Female
A: Control	0	35 (1 to 35)	35 (501 to 535)
B: Low dosage	25	35 (101 to 135)	35 (601 to 635)
C: Medium dosage	100	35 (201 to 235)	35 (701 to 735)
D: High dosage	400	35 (301 to 335)	35 (801 to 835)

6. Mating Method and Starting Day of Pregnancy

The mating period was limited to three weeks. First, male and female rats of the same dosage group were paired 1-to-1. Monitoring of copulatory behavior was done over a period of 14 days, defined as mating period I. For pairs that did not copulate, males were paired with untreated females, and females were paired with untreated males. Copulation was monitored for a further 7 days, defined as mating period II.

Copulation was confirmed by the presence of the vaginal plug or a positive vaginal smear. Day 0 of pregnancy was defined as the day of copulation.

7. Observation of Rats

7.1 Observation of the parent (P) generation of the rats

Clinical Signs and mortality were checked every day. Male rats were weighed twice per week.

Female rats were weighed twice per week during two weeks before the mating, every day during the mating period (not summed), on Days 0, 1, 7, 14 and 20 of pregnancy, and Days 1, 4, 7, 14 and 21 after parturition. Food intake of the male rats was measured once per week. Food intake of the female rats was measured once per week before mating, on Days 1, 7, 14 and 20 of pregnancy, and on Days 1, 4, 7, 14 and 21 after parturition.

To examine the estrous cycle of female rats, vaginal smears were examined from the beginning of dosing until copulation. Copulation index of male rats was calculated and the period up to copulation was recorded. Within about one week after copulation, male rats were sacrificed for postmortem examination. Testis, epididymis and prostate were weighed. Copulation index was also calculated for female rats. Ovary and uterus from female rats that did not successfully mate and from nonpregnant female rats were examined histopathologically.

7.2 Observation of P rats and fetuses of the caesarean section group

About 20 rats of the caesarean section group were sacrificed for postmortem examination in the afternoon of Day 20 of pregnancy. Caesarean section was performed and the number of corpora lutea was counted. The numbers of implants, live fetuses and dead embryos or fetuses were counted by dissecting the uterine wall opposite the wide ligament adhesion. Dead embryos and fetuses were classified as follows. Death in early pregnancy refers to the presence of an implantation site or placenta with a diameter less than 3 mm, death in mid pregnancy refers to the presence of placenta with a diameter larger than 3 mm and death in late pregnancy refers to a dead fetus larger than the diameter of the placenta.

Body weight and placental weight were recorded for live fetuses. Fetuses were examined externally (including oral cavity) and sexed. Approximately half of the fetuses were fixed in alcohol for skeletal examination and the remaining half were fixed in Bouin's solution for visceral examination.

The head and neck regions of the fetuses were examined according to Wilson's method (1965). Anomalies and positions of thoracic and abdominal organs, and the running of the intestines were examined with a stereoscopic dissecting microscope according to Barrow and Taylor's method (1969).

Skeletons of the fetuses were examined by preparing transparent skeletal specimens according to Dawson's method (1926). The state of ossification of cervical and caudal vertebrae, skeletal abnormalities and variations were described.

7.3 Observation of P rats in the delivery group

Clinical Signs and mortality were monitored daily. Abnormalities in parturition and lactation behaviors were evaluated during the perinatal period.

The P rats were sacrificed for postmortem examination after the end of lactation (Day 22 after parturition) and the number of implantation sites was recorded.

7.4 Observation of postnatal growth of F_1 rats

The numbers of live and dead neonates were counted within 24 hours of delivery. Live neonates were weighted, sexed, and examined for presence of external abnormalities. Dead neonates were fixed in Bouin's solution immediately after parturition.

Pups were culled at random on Day 4 after parturition to 8 per litter (4 males and 4 females, if possible). Culled pups were fixed in Bouin's solution. Remaining pups were lactated and Clinical Signs were observed Day 21 after parturition. Simultaneously, pinna detachment, incisor eruption, fur appearance, eye opening, external auditory canal opening, and gait on paws were examined. One male and one female pup from each litter were examined for surface righting, pinna reflex, pupillary reflex and pain response. Body weight was measured at 1, 4, 7, 14 and 21 days of age during the lactation period. After weaning, one male and one female pup were selected from each litter. Male and female animals were housed individually, and body weight and food intake were measured. The remaining rats were housed in separate cages by sex, with a maximum of 3 rats per cage to measure body weight only (not summed). Vaginal opening of female pups and penis formation of male pups were examined

in each litter. The completion of penis formation was defined as the time when the penis body becomes exposable.

Ten males and 10 females from each dosage group were subjected to a behavioral examination (open field test) at 5 weeks of age, and to a learning ability examination (water multiple T-maze test) at 6 weeks of age.

All but 4 rats, 2 males and 2 females, were sacrificed for postmortem examination at 7 weeks of age. One male and female pair from each litter was mated at 10 weeks of age to evaluate reproductive function. Rats that were separately housed for evaluation of body weight and food intake were sacrificed at 10 weeks of age.

One rat each from each litter was sacrificed at 7 and 10 weeks of age and the following organs were weighed: thymus, heart, liver, spleen, kidney, lung, adrenal glands, prostate gland, testis/ovary, brain, and pituitary gland. Relative organ weight was also calculated for these organs.

7.5 Reproductive function of F_1 rats

At 10 weeks of age, one male and one female, were selected from each litter. They were mated within the same dosage group, avoiding brother-sister mating. The mating period was limited to 3 weeks and the mating method was similar to that for the P rats.

Body weight of female F_1 rats after copulation was measured on Days 0, 1, 4, 7, and 14 of pregnancy. Food intake was measured on Days 1, 4, 7, and 14 of pregnancy. These females were sacrificed for postmortem examination on Day 14 of pregnancy. The number of corpora lutea, implantations, live and dead fetuses, and the number of resorbed embryos were counted.

Male F_1 rats used for mating were sacrificed for postmortem examination within 7 days after mating to measure the weight of testis, epididymis and prostate.

8. Statistical Methods

To examine significant differences of the test values, Fisher's exact test was applied to Clinical Signs, mortality, copulation index, fertility index, gestation index, surface righting, pinna reflex, pupillary reflex, pain response and the findings from postmortem examination. Analysis of variance was performed on other items and if declared significant ($P < 0.05$), Dunnett's t-test was applied.

Differences were judged as significant at $P < 0.05$.

9. Storage of Data and Specimens

The protocol, all data, the final report, and specimens are to be stored for 5 years after the end of the study. Retention beyond this point will be by consultation with the sponsor.

Results

I. Observations of Parent (P) Rats

1. Clinical Signs and Mortality

1.1 Male rats (Tables 1 and 2)

No abnormal clinical sign or death was observed among the 35 rats in the control group. One male rat of 35 in the 25 mg/kg group died in the 4th week (before mating) without showing any abnormal clinical signs. No abnormal clinical sign or death was observed in the remaining 34 male rats. No abnormalities related to the test substance were observed in the 35 animals each in the 100 and 400 mg/kg groups.

1.2 Female rats (Tables 3 and 4)

No abnormalities related to the test substance were observed in any of the 35 female rats of the control, 25, 100 and 400 mg/kg groups throughout the test period.

2. Body Weight

2.1 Male rats (Table 5)

Before mating, body weights of male rats in the treatment groups were similar to the control group.

2.2 Female rats (Tables 6, 7 and 8)

Before mating, body weights of female rats in the treatment groups were similar to the control group. On Day 20 of pregnancy, body weights in the 25 and 100 mg/kg groups were significantly lower. No significant difference was observed in the 400 mg/kg group. Throughout the lactation period, body weights of the treatment groups were similar to the control group.

3. Food intake

3.1 Male rats (Table 9)

Food intake of the 25 mg/kg group was similar to the control group. Food intake was slightly lower on Days 51 and 58 for the 100 mg/kg group and on Days 2 and 58 for the 400 mg/kg group. Significant differences from the control group were seen in both groups.

3.2 Female rats (Tables 10, 11 and 12)

Before mating and during pregnancy, food intake of female rats in the treatment groups was similar to the control group. During lactation, food intake of the 25, 100, and 400 mg/kg groups was similar to the control group.

4. Copulation and Fertility Results

4.1 Copulation and fertility indices of male P rats (Table 13)

Copulation indices of the treatment groups were from 94.3 to 100%, and fertility indices were from 93.9 to 100%. No significant difference from the control group was seen.

One male rat in the 25 mg/kg group and two male rats in the 100 mg/kg group did not copulate during mating period I, but did copulate with untreated female rats during mating

period II. Two male rats in the control group, two in the 25 mg/kg group, and another two in the 400 mg/kg group failed to become pregnant, but no abnormality was found in their reproductive organs at postmortem examination.

4.2 Estrous cycle, copulation and fertility indices of female P rats (Table 14)

Estrous cycles of the female rats in the treatment groups were similar to the control group and no significant difference was observed.

Copulation indices of the treatment groups were from 94.3 to 100% and fertility indices were from 94.1 to 100%. No significant difference from the control group was seen.

5. Reproductive Findings

5.1 Ovulation, implantation, and fetal development (Table 15)

No significant difference was observed between the treatment groups and the control group for the numbers of ovulations (number of corpora lutea) and implants, implantation rate, embryolethality (rate) and the number of live fetuses.

Body weights of male and female fetuses of the 25 and 100 mg/kg groups were similar to the control group. While no significant difference was observed in body weight of male fetuses of the 400 mg/kg group, body weight of female fetuses was significantly lower than the control group. Placental weights in the treatment groups were similar to the control group.

Sex ratios (% male) in the treatment groups were similar to the control group.

5.2 Morphological examinations of live fetuses

5.2.1 External examination of fetuses (Table 16)

One fetus from each of 3 dams in the control group showed anomalies. Two fetuses had absence of tail and one fetus had a unilateral anophthalmia. No anomaly was observed in the fetuses of the 25, 100, and 400 mg/kg groups.

5.2.2 Visceral examination of fetuses (Table 17)

Hydronephrosis (hypoplasia of renal papilla) was observed in 2, 5, and 2 fetuses of each of 2 dams of the control, 100, and 400 mg/kg groups, respectively. No anomaly was observed in the fetuses of the 25 mg/kg group.

No significant difference in the incidence of fetuses with anomalies was observed.

5.2.3 Skeletal examination of fetuses (Table 18)

Degree of ossification:

The numbers of ossified bodies of cervical vertebrae and coccygeal bones, used as the indices of ossification, were similar between the control and the treatment groups and no significant difference was observed.

Skeletal variations:

No significant difference was observed in the incidences of cervical ribs, lumbar ribs, shortening of 13th ribs, 12 thoracic vertebrae, 5 lumbar vertebrae, and 7 lumbar vertebrae between the control and treatment groups.

Skeletal anomalies:

Wavy ribs were observed in one fetus in the 25 mg/kg group. Sacralisation (unilateral) was observed on one fetus of the control group, and lumbarisation (unilateral) was observed on one fetus in the 25 mg/kg group. No anomaly was observed on the fetus in the 100 and 400 mg/kg groups. No significant difference in the incidence of fetuses with skeletal anomalies was observed.

6. Examination of Female P Rats at Delivery (Table 19)

All of the pregnant female rats of the control and treatment groups delivered F_1 pups normally and the birth indices were 100%.

No abnormalities were observed in the 13, 11, 12, and 12 P rats of the control, 25, 100, and 400 mg/kg group, respectively.

7. Postmortem Examination of Parent (P) Rats

7.1 Gross findings in male P rats (Table 20)

No abnormalities were observed in the 35 rats of the control group. One rat in the 25 mg/kg group that died in the 4th week showed cyanoses in the limbs, ulcer of the scrotum, congestion of liver, kidney and lung, patchy hemorrhage of thymus, and adhesion of the epididymis to the ulcerated scrotum. No abnormality was observed in the remaining 34 rats in this group. One rat in the 100 mg/kg group was observed to have bilateral atrophy of testis. No abnormality was observed in the remaining 34 rats in the group. No abnormality was observed in 35 rats in the 400 mg/kg group.

7.2 Gross findings in female P rats (Tables 21 and 22)

Necropsy on Day 20 of pregnancy:

One rat in the control group was observed to have unilateral hydronephrosis. No abnormality was observed in the remaining 19 rats in the same group. No significant lesion was observed in 21 rats of the 25 mg/kg group and 21 of the 100 mg/kg group. Three rats of the 400 mg/kg group were observed to have subcutaneous hemorrhage in the back but no abnormality was observed in the remaining 18.

Necropsy after lactation:

No significant lesion was observed in 13 rats of the control group, 11 rats of the 25 mg/kg group, 12 rats of the 100 mg/kg group, and 12 rats of the 400 mg/kg group.

7.3 Organ weights in male P rats (Tables 23 and 24)

Absolute and relative organ weights of reproductive organs were determined in male rats

sacrificed for postmortem examination upon completion of mating. Absolute and relative organ weights of testis, prostate, and epididymis of the treatment group were similar to the control group and no significant difference was observed.

7.4 Histopathological examinations

No significant lesion was observed in histopathological examination of the dead male rat (No. 114) in the 25 mg/kg group. A male rat (No. 223) in the 100 mg/kg group showed atrophy of testis at necropsy. Histopathological examination of the testis revealed interstitial edema, atrophy of seminiferous tubule and the decrease of spermatogenesis. A histopathological examination of reproductive organs conducted on the non-copulating or non-pregnant 2 female rats (Nos. 512 and 524) in the control group, 3 female rats (Nos. 607, 617 and 629) in the 25 mg/kg group, and 2 female rats (Nos. 807 and 822) in the 400 mg/kg group showed no abnormality.

II. Observations of Neonatal (F_1) Rats

1. Number of F_1 Pups (within 24 hours of birth) (Table 25)

Mean numbers of F_1 pups of the treatment groups, 14.2 to 14.7, were similar to the control group (15.2). No significant difference was seen.

Sex ratio (male %) of F_1 pups showed no significant difference between control and treatment groups.

2. Observation of Growth of F_1 Rats

2.1 Viability and weaning indices (Table 26)

Mean numbers of live F_1 rats of the treatment groups during the lactation period were similar to the control group and no significant difference was observed.

Viability indices at 4 days of age were high (96.0 to 100%) for both control and treatment groups, and no significant difference was observed.

Weaning indices at 21 days of age were high (98.9 to 100%) for both control and treatment groups, and no significant difference was observed.

2.2 Body weight of F_1 pups during the lactation period (Table 27)

Body weight changes of both male and female pups of the treatment groups were similar to the control group, and no significant difference was observed.

2.3 Behavioral development of F_1 pups (Tables 28 and 29)

Positive rate of surface righting at 2 days of age, gait on paws at 14 days of age, pinna reflex at 18 days of age, pain response and pupillary reflex at 21 days of age were similar to the control group and no significant difference was observed.

2.4 Physical development of F_1 pups (Tables 30 and 31)

Proportion of pinna detachment and fur appearance by 3 days of age were high and were similar to the control group. No significant difference was seen. Proportion of incisor eruption by 10 days of age and external auditory canal opening by 12 days of age showed no significant difference between the control and the treatment groups. Proportion of male rats with eye opening by 14 days of age showed no significant difference between treatment and control groups.

Days to penis formation in the treatment groups were similar to the control group and no significant difference was observed. Days to vaginal opening were similar between control and treatment groups, and no significant difference was observed.

3. Observation of F_1 Rats after Weaning

3.1 Clinical Signs and mortality

Male rats (Tables 32 and 33):

No symptom (abnormality) was observed in male F_1 rats in either control or treatment groups. One male rat among 42 in the 25 mg/kg group died at 10 weeks of age, and one male rat among 48 in the 400 mg/kg group died during the mating period (11 weeks of age). Neither rat showed any symptoms. No male rat died in the control and the 100 mg/kg groups.

Female rats (Tables 34 and 35):

No symptom (abnormality) was observed in female F_1 rats in the control, 25 and 100 mg/kg groups. One female rat among 47 in the 400 mg/kg group showed hemophthalmia of the left eye from 6 weeks of age. No mortality was observed in female rats of either control or treatment groups.

3.2 Body weight of F_1 rats

Male rats (Table 36):

Body weight gains in the 25, 100, and 400 mg/kg groups were similar to the control group, and no significant difference was observed.

Female rats (Tables 37 and 38):

Body weight gains in the treatment groups were similar to the control group throughout neonatal development and pregnancy, and no significant difference was observed.

3.3 Food intake of F_1 rats

Male rats (Table 39):

Food intake in the treatment groups was similar to the control group, and no significant difference was observed.

Female rats (Tables 40 and 41):

Food intake in the treatment groups was similar to the control group throughout neonatal development and pregnancy, and no significant difference was observed.

4. Behavioral Test

The open field test was conducted on ten males and ten females at 5 weeks of age.

Male rats (Table 42):

A significant increase in latency was observed in the 25 mg/kg group in the 3rd trial, but latencies in the 100 and 400 mg/kg groups showed no significant difference from the control group. Grooming numbers of the treatment groups were similar to the control group.

Sniffing decreased in the 2nd trial in the 25 mg/kg group and a significant difference was observed from the control group. No significant difference from the control group was observed for the number of sniffings of the 100 and 400 mg/kg groups.

Rearing, defecation and urination in the treatment groups were similar to the control group and no significant difference was observed.

Female rats (Table 43):

Latencies in the 25, 100, and 400 mg/kg groups were similar to the control group and no significant difference was observed. Grooming numbers in the treatment groups were similar to the control group and no significant difference was observed. The number of sniffings in the control and 25 mg/kg groups were similar and no significant difference was observed. The number of sniffings in the 100 mg/kg group also showed no significant difference from the control group. The number of sniffings decreased significantly in the 1st trial in the 400 mg/kg group. The number of rearing significantly increased in the 3rd trial in the 25 mg/kg group. Rearings in the 100 and 400 mg/kg groups were similar to the control group and no significant difference was observed. Defecation was similar between groups, and no significant difference was observed. Urination in the 25 mg/kg group decreased significantly in the 1st trial, but was similar to the control group in the 2nd and 3rd trials. Urination in the 100 and 400 mg/kg groups was similar to the control group, and no significant difference was observed. Ambulation in the control and the treatment groups was similar, and no significant difference was observed.

5. Water Multiple T-Maze Learning Test

Male rats (Tables 44 and 45):

Latencies in the 25, 100 and 400 mg/kg groups were similar to the control group and no significant difference was observed.

The numbers of errors in the 25 and 100 mg/kg groups were both 0 in the 4th trial on the 3rd day. The number of errors in the 400 mg/kg group was similar to the control group and no significant difference was observed.

Female rats (Tables 46 and 47):

Latencies in the 25, 100 and 400 mg/kg groups were similar to the control group and no significant difference was observed.

The number of errors in each treatment group was similar to the control group and no significant difference was observed.

6. Copulation and Fertility Indices of F_1 Rats

Male rats (Table 48):

Copulation indices in the treatment groups were 100% and fertility indices were 91.7 to 100%. No significant difference from the control group was seen.

Female rats (Table 49):

Copulation indices and fertility indices in the treatment groups were 100 and 91.7 to 100%, respectively, and no significant difference was observed from the control group.

7. Ovulation, Implantation, and Embryo Survival at Day 14 of Pregnancy of F_1 Rats

(Table 50)

The numbers of ovulations (number of corpora lutea), implants, dead and live embryos, and the rates of implantation, embryolethality and survival in the treatment groups were similar to the control group, and no significant difference was observed.

8. Postmortem Examination of F_1 Rats

8.1 Necropsy at 7 weeks of age

8.1.1 Gross findings

Male rats (Table 51):

No abnormality was observed in 26 male rats in the control group, 20 in the 25 mg/kg group, 25 in the 100 mg/kg group, and 24 in the 400 mg/kg group.

Female rats (Table 52):

No abnormality was observed in 26 female rats in the control group, 23 in the 25 mg/kg group, 23 in the 100 mg/kg group and 23 in the 400 mg/kg group.

8.1.2 Organ weights

Male rats (Tables 53 and 54):

In the 25 mg/kg group, a significant increase was observed in absolute and relative organ weights for heart. Absolute and relative organ weights in the 100 and 400 mg/kg groups were similar to the control group and no significant difference was observed.

Female rats (Tables 55 and 56):

Absolute and relative organ weights in the 25 and 100 mg/kg groups were similar to the control group and no significant difference was observed. Absolute and relative organ weights of the left ovary of the 400 mg/kg group were significantly lower, but no significant difference was observed with the total absolute and relative organ weights of the left and right ovaries.

8.2 Necropsy at 10 weeks of age

8.2.1 Gross findings

Male rats (Table 57):

No abnormality was observed in the 13 male rats in the control group, 10 in the 25 mg/kg group, 12 in the 100 mg/kg group, and 12 in the 400 mg/kg group.

Female rats (Table 58):

No abnormality was observed in the 13 female rats in the control group, 11 in the 25 mg/kg group, and 12 in the 100 mg/kg group. One female rat in the 400 mg/kg group showed unilateral (left side) hemophthalmia, but the remaining 11 rats had no abnormality.

8.2.2 Organ weights

Male rats (Tables 59 and 60):

Absolute and relative organ weights in the treatment groups were similar to the control group and no significant difference was observed.

Female rats (Tables 61 and 62):

Absolute and relative organ weights in the 25 and 100 mg/kg groups were similar to the control group and no significant difference was observed. Absolute and relative organ weights of the left ovary of the 400 mg/kg group were significantly lower.

8.3 Postmortem examinations of F_1 rats used for evaluation of reproductive function

8.3.1 Gross findings

Mate rats (Table 63):

Animals were sacrificed for postmortem examination within 7 days after copulation. Rats scheduled for mating to examine reproductive functions at weaning but that died beforehand were included.

No abnormalities were observed in 13 male rats of the control group. One of 12 rats in the 25 mg/kg group died at 10 weeks of age. It had congestion in liver and lung. Bilateral atrophy of testis was observed in one male rat in the 100 mg/kg group, but the remaining 11 rats had no abnormality. One of 12 rats in the 400 mg/kg group died at 11 weeks of age. It was observed to have cyanoses of the limbs, congestion and turbidity of liver, congestion of lung, and patchy hemorrhage of thymus. Another male rat was observed to have swelling of testis. No abnormality was observed among the remaining 10 rats.

Female rats (Table 64):

Female F_1 rats were sacrificed after mating for postmortem examination on Day 14 of pregnancy. No abnormality was observed in the 12 female rats of the control group, 11 in the 25 mg/kg group, 11 in the 100 mg/kg group or 12 in the 400 mg/kg group.

8.3.2 Organ weights in male F_1 rats (Tables 65 and 66)

Absolute and relative organ weights of the testis, prostate and epididymis in the treatment groups were similar to the control group and no significant difference was observed.

8.3.3 Histopathological examinations

Histopathological examinations of the two rats that died, one male rat (No. 6045) in the 25 mg/kg group and one male rat (No. 8265) in the 400 mg/kg group, showed congestion of liver and lung, and edema of lung, but no other remarkable changes were observed. Atrophy of testis was found on one male rat (No.7295). Histopathological findings included atrophy of the seminiferous tubule and decrease of spermatogenesis. One of the female rats (No. 5201) in the control group did not become pregnant, but its reproductive organs showed no abnormality. One female rat (No. 7261) in the 100 mg/kg group mated with a male rat (No. 7295) of the same group but it did not become pregnant. No abnormality was observed in the reproductive organs of this female rat upon histopathological examination.

Discussion

To test the effects on the reproductive functions of rats, Arbutin was subcutaneously injected into CD rats at dose levels of 25, 100 and 400 mg/kg/day. Control group was given vehicle (physiological saline).

Food intake of male P rats in the 100 mg/kg group decreased significantly during the 8th and 9th weeks, and in the 400 mg/kg group during the 1st and 9th weeks. Body weight changes of the male P rats of each treatment group were similar to the control group. Death of a male P rat in the 25 mg/kg group was regarded as an incidental death without any association with the test substance. During the postmortem examinations, no effect of test substance was observed on the reproductive organs of the male P rats. Food intake of the female P rats was similar to the control group. Body weight changes of the female P rats in the 25 and 100 mg/kg groups showed a transient decrease on Day 20 of pregnancy, but the 400 mg/kg

group showed similar body weight changes to the control group throughout the test period. No effect of the test substance was observed on the estrous cycle, copulation index or fertility index of the female P rats.

No effect of the test substance was observed on the number of implants, dead embryos and fetuses, live fetuses, or on implantation rate or embryo lethality rate. A significant decrease in body weight of female fetuses was observed in the 400 mg/kg group, but no significant difference was observed for male fetuses. Placental weight, sex ratio, incidences of fetuses with external and internal organ anomalies, skeletal ossification, and incidences of fetuses with skeletal variations and anomalies were similar between treatment and control groups. No effect of test substance was observed. No effect of the test substance was observed on delivery and gestation indices of the female P rats. No abnormality was observed in P rats sacrificed for postmortem examinations after their offspring were weaned (Day 22 after parturition).

No effect of the test substance was observed on the number of births, sex ratio, viability index at 4 days of age, weaning index at 21 days of age, body weight gains before weaning, or behavioral or physical development of F_1 rats.

No abnormality was observed in Clinical Signs of F_1 rats after weaning. One male rat from the 25 mg/kg group and one male rat from the 400 mg/kg group died at 10 and 11 weeks of age, respectively. Congestion of liver and lung were common gross findings and no association with dosage was observed. These deaths were not attributed to the test substance and were considered incidental.

No effect of the test substance was observed on body weight and food intake from 3 to 10 weeks of age.

In the open field test conducted at 5 weeks of age, longer latency on the 3rd trial and fewer sniffings in the 2nd trial were seen in the male F_1 rats of the 25 mg/kg group. In addition, female F_1 rats of the same group showed more rearings in the 3rd trial and fewer urinations in the 1st trial. These data did not show a dose response relation and were not thought to be the effects of the test substance. The female F_1 rats of the 400 mg/kg group showed fewer sniffings in the 1st trial, but no significant difference from the control group was observed in the 2nd and 3rd trials. These differences were not considered to be the effects of the test substance.

In a learning test (water multiple T-maze test) in male and female F_1 rats at 6 weeks of age, no effect of the test substance was observed on latencies or the number of errors.

In the reproductive performance examinations in male and female F_1 rats, no effect of the test substance was observed on the copulation index and fertility index. In pregnant F_1 dams in the treatment groups, no effect of the test substance was observed on the number of corpora lutea (ovulation number), the number of implants and implantation rate, the number of dead embryos, embryolethality rate, the number of live fetuses or survival rate.

In the postmortem examination of F_1 rats at 7 weeks of age, an increase in the absolute and relative organ weights of heart was observed in male rats in the 25 mg/kg group. This trend was not observed at higher doses and the changes were not thought to be the effects of the test substance. Decreased absolute and relative organ weights were observed in left ovary of female F_1 rats in the 400 mg/kg group.

Postmortem examination of F_1 male rats of 10 weeks of age showed no abnormality attributable to the test substance. For female F_1 rats of the 400 mg/kg group, similar to the findings at 7 weeks of age,

showed a decrease in absolute and relative organ weights of the left ovary. The weight of right ovary and the total weight of left and right ovaries of the female F_1 rats of both 7 and 10 weeks of age of the same group are similar to the control group, and no significant difference was observed between the groups.

In addition, the mean number of ovulations (the number of corpora lutea) of the same group was similar to the control group and the reproductive functions were normal. No effect of the test substance was observed on the functions of the ovaries. In other words, 400 mg/kg/day of the test substance had no effect on the reproductive functions of the male and female F_1 rats.

Postmortem examination of the male F_1 rats after the mating period and the female F_1 rats on Day 14 of pregnancy showed no abnormality caused by the test substance.

In summary, these data indicate that subcutaneous injection of Arbutin at 400 mg/kg/day from before mating to the end of mating for males and from before conception to the end of lactation period for females affects food intake of the male P rats, fetal body weight (female), and absolute and relative organ weights of the left ovary as observed in the postmortem examinations of female F_1 rats at 7 and 10 weeks of age. A decrease in the food intake was also observed at 100 mg/kg/day in male P rats before mating.

Based on these results, it is concluded that 400 mg/kg/day of Arbutin does not affect reproductive functions of the parent animals and F_1 rats, but caused body weight decrease in female fetuses, decreased organ weights of the unilateral ovary of female F_1 rats. In conclusion, the no observable effect dose of Arbutin was estimated to be 100 mg/kg/day.

Table 1 Clinical Signs in Male P Rats

	Number of animals with abnormalities			
	a 0	25	100	400
Clinical Signs	b 35	35	35	35
Abnormal findings	0	0	0	0

a: Dose (mg/kg/day)
b: Number of animals observed

Table 2 Mortality in Male P Rats

Dose (mg/kg/day)	Number of animals	Number of deaths		Mortality (%)
		Before mating	After mating	
0	35	0	0	0
25	35	1	0	2.9
100	35	0	0	0
400	35	0	0	0

Table 3 Clinical Signs in Female P Rats

	Number of animals with abnormalities			
	[a] 0	25	100	400
Clinical Signs	[b] 35	35	35	35
Abnormal findings	0	0	0	0

a: Dose (mg/kg/day)
b: Number of animals observed

Table 4 Mortality in Female P Rats

Dose (mg/kg/day)	Number of animals	Number of deaths			Mortality (%)
		Before mating	During pregnancy	During lactation	
0	a22	0	0		0
	b13	0	0	0	0
25	a23	0	0		0
	b12	0	0	0	0
100	a23	0	0		0
	b12	0	0	0	0
400	a22	0	0		0
	b13	0	0	0	0

a: Necropsy at the end of pregnancy
b: Delivery

Table 5 Body Weight of Male P Rats

Dose (mg/kg/day)	Number of animals	Body weight (g)										
		0	1	4	7	11	14	18	21	25	28	32
0	35	197 8	206 8	233 10	259 13	293 15	318 17	347 20	367 23	391 24	407 25	429 28
25	35	197 9	205 9	231 11	257 13	291 15	316 18	344 22	363 24	385 27	402 29	422 32
100	35	197 8	205 9	231 10	258 12	293 15	318 18	345 22	363 25	386 28	401 30	420 33
400	35	197 8	204 9	231 11	257 12	291 15	316 16	344 20	361 22	384 24	400 26	419 28

Dose (mg/kg/day)	Number of animals	Body weight (g)									
		35	39	42	46	49	53	56	60	63	(days)
0	35	442 29	458 31	473 34	486 35	497 36	509 39	518 41	529 43	535 44	
25	35	436 34	452 35	465 38	478 39	489 40	496 43	505 45	516 45	525 47	
100	35	434 35	449 38	462 39	475 40	488 41	496 42	504 43	516 45	524 46	
400	35	434 29	450 31	463 32	477 34	488 36	497 37	505 37	517 38	524 38	

Values are mean and standard deviation

Table 6 Body Weight of Female P Rats

Dose (mg/kg/day)	Number of animals	Body weight (g) 0	1	4	7	11	14 (days)
0	35	192 7	200 8	206 8	214 10	226 12	233 12
25	35	192 7	198 8	205 8	212 8	223 10	227 11
100	35	192 7	198 9	205 9	213 10	226 11	233 12
400	35	193 7	197 8	205 8	214 9	226 11	234 11

Values are mean and standard deviation

Table 7 Body Weight of Pregnant Rats

Dose (mg/kg/day)	Number of animals	Body weight (g) 0	1	7	14	20 (days)
0	33	242 14	251 15	279 15	318 14	401 18
25	32	235 12	244 12	273 14	310 16	386* 18
100	33	244 12	250 13	278 13	313 16	387* 23
400	33	243 13	252 13	281 15	317 20	389 31

Values are mean and standard deviation
*: $P < 0.05$

Table 8 Body Weight of Rats During Lactation

Dose (mg/kg/day)	Number of animals	Body weight (g) 1	4	7	14	21 (days)
0	13	296 14	324 15	326 16	337 14	314 13
25	11	296 19	316 16	324 15	337 11	314 16
100	12	299 23	321 20	326 20	342 15	320 15
400	12	293 24	321 23	330 20	336 14	314 12

Values are mean and standard deviation

Table 9 Food Intake of Male P Rats

Dose (mg/kg/day)	Number of animals	Food intake (g) 2	9	16	23	30	37	44	51	58 (days)
0	35	25 2	29 2	31 3	31 3	30 3	30 3	32 4	31 3	31 3
25	35	24 2	30 2	32 3	32 3	31 3	31 3	32 3	30 3	30 4
100	35	24 2	29 2	31 3	30 3	31 3	31 3	31 3	29* 3	29** 2
400	35	23** 2	29 3	30 3	31 2	31 3	31 3	30 3	30 3	28** 3

Values are mean and standard deviation
* : P<0.05
** : P<0.01

Table 10 Food Intake of Female P Rats

Dose (mg/kg/day)	Number of animals	Food intake (g)		
		2	9	(days)
0	35	19 2	19 2	
25	35	18 2	19 3	
100	35	18 2	20 2	
400	35	18 2	19 3	

Values are mean and standard deviation

Table 11 Food Intake of Pregnant Rats

Dose (mg/kg/day)	Number of animals	Food intake (g)				
		1	7	14	20	(days)
0	33	22 2	25 3	25 3	26 4	
25	32	23 2	24 3	26 3	26 3	
100	33	23 3	24 2	24 2	26 3	
400	33	22 2	25 4	25 4	26 3	

Values are mean and standard deviation

Table 12 Food Intake of Rats During Lactation

Dose (mg/kg/day)	Number of animals	Food intake (g)					
		1	4	7	14	21	(days)
0	13	18 9	50 5	48 5	62 4	75 8	
25	11	18 10	46 6	50 4	64 7	79 9	
100	12	23 4	46 6	49 6	65 5	74 5	
400	12	13 9	46 6	47 5	61 6	74 8	

Values are mean and standard deviation

Table 13 Copulation and Fertility Indices of Male P Rats

Dose (mg/kg/day)	Number of males mated	Number of males copulating successfully	Copulation Index (%)	Number of fertile males	Fertility Index (%)
0	35	35	100	33	94.3
25	34	33	97.1	31	93.9
100	35	33	94.3	33	100
400	35	35	100	33	94.3

Copulation Index = (number of males copulating successfully/number of males mated) × 100 (%)
Fertility Index = (number of males having fertilized females/number of males copulating successfully) × 100 (%)

Table 14 Estrous Cycle, Copulation and Fertility Indices of Female P Rats

Dose (mg/kg/day)	Number of females mated	Estrous cycle [a] (days)	Number of females copulating successfully	Copulation Index (%)	Number of pregnant females	Fertility Index (%)
0	35	4.2 0.5	35	100	33	94.3
25	35	4.2 0.3	34	97.1	32	94.1
100	35	4.1 0.4	33	94.3	33	100
400	35	4.2 0.5	35	100	33	94.3

a: Values are mean and standard deviation
Copulation Index = (number of females copulating successfully/number of females mated) × 100 (%)
Fertility Index = (number of pregnant females/number of females copulating successfully) × 100 (%)

Table 15 Ovulation, Implantation and Fetal Development

Dose (mg/kg/day)	Number of animals	Number of corpora lutea	Number of implants	Implantation rate (%)	Number of dead embryos or fetuses				Embryolethality rate (%)	Number of live fetuses			Body weight (g)		Placental weight (mg)		Sex ratio (Male %)
					Early	Middle	Late	Total		Male	Female	Total	Male	Female	Male	Female	
0	20	(372)	(314)		(10)	(0)	(0)	(10)		(146)	(158)	(304)					
		18.6	15.7	86.0	0.5	0.0	0.0	0.5	3.2	7.3	7.9	15.2	3.64	3.52	486	470	48.7
		3.6	2.3	12.4	0.6	0.0	0.0	0.6	3.9	1.7	2.6	2.4	0.39	0.32	59	57	12.8
25	21	(380)	(310)		(17)	(2)	(0)	(19)		(154)	(137)	(291)					
		18.1	14.8	83.5	0.8	0.1	0.0	0.9	5.9	7.3	6.5	13.9	3.67	3.53	488	484	52.7
		3.1	2.1	16.1	1.0	0.3	0.0	1.0	7.4	2.2	2.1	2.0	0.36	0.32	91	124	15.7
100	21	(371)	(310)		(23)	(2)	(0)	(25)		(146)	(139)	(285)					
		17.7	14.8	84.7	1.1	0.1	0.0	1.2	7.9	7.0	6.6	13.6	3.73	3.54	481	460	51.6
		2.4	2.7	16.8	1.4	0.4	0.0	1.5	10.4	2.3	2.5	2.9	0.22	0.19	41	43	13.9
400	21	(354)	(299)		(20)	(4)	(0)	(24)		(125)	(150)	(275)					
		16.9	14.2	82.7	1.0	0.2	0.0	1.1	7.2	6.0	7.1	13.1	3.45	3.23*	497	488	43.3
		3.0	4.7	26.2	0.9	0.6	0.0	1.3	7.9	3.2	3.2	4.5	0.47	0.48	89	90	18.6

Values are mean and standard deviation
(Parentheses) indicates a total

Table 16 External Examination of Fetuses

Dose (mg/kg/day)	Number of dams	Number of Fetuses examined	Absence of tail (%)	Anophthalmia (%)	Number of abnormal fetus (%)
			(2)	(1)	(3)
0	20	304	0.7	0.4	1.0
			2.1	1.6	2.5
			(0)	(0)	(0)
25	21	291	0	0	0*
			0	0	0
			(0)	(0)	(0)
100	21	285	0	0	0*
			0	0	0
			(0)	(0)	(0)
400	21	275	0	0	0*
			0	0	0

Values are mean and standard deviation
(Parentheses) indicate the number of fetuses with anomalies
*: P<0.05

Table 17 Visceral Examination of Fetuses

Dose (mg/kg/day)	Number of dams	Number of fetuses examined	Hydronephrosis (%)				Number of fetuses with anomalies (%)
			Unilateral		Bilateral	Total	
			Left	Right			
0	20	147	(1) 0.6 2.8	(1) 0.5 2.2	(0) 0 0	(2) 1.1 3.5	(2) 1.1 3.5
25	21	141	(0) 0 0	(0) 0 0	(0) 0 0	(0) 0 0	(0) 0 0
100	21	138	(0) 0 0	(3) 2.0 6.4	(2) 1.2 5.5	(5) 3.2 11.3	(5) 3.2 11.3
400	20	132	(1) 0.7 3.2	(1) 0.7 3.2	(0) 0 0	(2) 1.4 4.4	(2) 1.4 4.4

Values are mean and standard deviation
(Parentheses) indicate the number of fetuses with anomalies

Table 18 Skeletal Examination of Fetuses

Dose (mg/kg/day)	Number of dams	Number of fetuses examined	Degree of ossification		Variations (%)								
					Cervical ribs		Lumbar ribs				Shortening of 13th rib		
					Unilateral	Total	Unilateral		Bilateral	Total	Unilateral		Total
			Cervical corpus vertebrae	Coccygeal bones	Right		Left	Right			Left	Right	
0	20	157	1.0	4.1	(0)	(0)	(1)	(0)	(4)	(5)	(2)	(0)	(2)
					0	0	0.7	0	2.4	3.1	1.1	0	1.1
			1.1	0.4	0	0	3.2	0	7.9	8.4	5.0	0	5.0
25	21	150	0.9	4.0	(1)	(1)	(2)	(1)	(1)	(4)	(0)	(0)	(0)
					0.6	0.6	1.4	0.7	0.7	2.7	0	0	0
			0.6	0.7	2.7	2.7	4.3	3.1	3.1	5.8	0	0	0
100	21	147	0.9	4.2	(1)	(1)	(4)	(3)	(1)	(8)	(1)	(1)	(2)
					0.7	0.7	3.0	1.9	1.0	5.9	0.6	0.8	1.4
			0.6	0.4	3.1	3.1	9.2	6.1	4.4	15.2	2.7	3.6	4.4
400	21	143	0.6	3.4	(0)	(0)	(1)	(1)	(2)	(4)	(0)	(0)	(0)
					0	0	0.5	0.7	1.2	2.4	0	0	0
			0.5	1.5	0	0	2.2	3.1	3.9	5.1	0	0	0

Values are mean and standard deviation
(Parentheses) indicate the number of fetuses with variations

Table 18 (continued) Skeletal Examination of Fetuses

Dose (mg/kg/day)	Number of dams	Number of fetuses examined	Variations (%)				Anomalies (%)			
			12 thoracic vertebrae	5 lumbar vertebrae	7 lumbar vertebrae	Number of fetuses with variations	Wavy ribs	Sacralisation	Lumbarisation	Number of fetuses with anomalies
0	20	157	(3) 1.7 7.4	(2) 1.1 3.4	(0) 0 0	(9) 5.3 12.4	(0) 0 0	(1) 0.6 2.5	(0) 0 0	(1) 0.6 2.5
25	21	150	(0) 0 0	(2) 1.9 8.7	(0) 0 0	(7) 5.2 10.0	(1) 0.6 2.7	(0) 0 0	(1) 1.0 4.4	(2) 1.5 5.0
100	21	147	(0) 0 0	(0) 0 0	(1) 0.5 2.4	(11) 7.9 15.9	(0) 0 0	(0) 0 0	(0) 0 0	(0) 0 0
400	21	143	(1) 0.5 2.4	(0) 0 0	(0) 0 0	(4) 2.4 5.1	(0) 0 0	(0) 0 0	(0) 0 0	(0) 0 0

Values are mean and standard deviation
(Parentheses) indicate the number of fetuses with variations or anomalies

Table 19 Examination of Female P Rats at Delivery

Dose (mg/kg/day)	Number of animals	Number of pregnant animals	Number of dams with live newborns	Gestation index (%)
0	13	13	13	100
25	12	11	11	100
100	12	12	12	100
400	13	12	12	100

Gestation index = (number of dams with live newborns/number of pregnant animals) × 100 (%)

Table 20 Gross Findings in Male P Rats

	Number of animals with abnormalities			
	[a] 0	25	100	400
Findings	[b] 35	35	35	35
Appearance				
Cyanoses of limbs	0	1 [c]	0	0
Ulcer of scrotum	0	1 [c]	0	0
Liver				
Congestion	0	1 [c]	0	0
Kidney				
Congestion	0	1 [c]	0	0
Lung				
Congestion	0	1 [c]	0	0
Thymus				
Patchy Hemorrhage	0	1 [c]	0	0
Testicle				
Bilateral atrophy	0	0	1	0
Epididymus				
Adhesion to ulcerated scrotum	0	1 [c]	0	0

a: Dose (mg/kg/day)
b: Number of animals examined
c: Rat No.114 (died in the 4th week)

Table 21 Gross Findings in Female P Rats (Day 20 of Pregnancy)

	Number of animals with abnormalities			
	[a] 0	25	100	400
Findings	[b] 20	21	21	21
Subcutaneous hemorrhage in back	0	0	0	3
Kidney				
Unilateral hydronephrosis	1	0	0	0

a: Dose (mg/kg/day)
b: Number of animals examined

Table 22 Gross Findings in Female P Rats (Post lactation)

	Number of animals with abnormalities			
	[a] 0	25	100	400
Findings	[b] 13	11	12	12
Abnormal findings	0	0	0	0

a: Dose (mg/kg/day)
b: Number of animals examined

Table 23 Absolute Organ Weights in Male P Rats

Dose (mg/kg/day)	Number of animals	Body weight (g)	Testis (g)			Prostate (mg)	Epididymis (mg)		
			Left	Right	Total		Left	Right	Total
0	35	544 44	1.64 0.12	1.63 0.12	3.27 0.23	1202 265	622 59	628 57	1250 106
25	34	534 48	1.62 0.12	1.62 0.13	3.24 0.24	1322 248	618 61	636 65	1254 119
100	35	534 47	1.61 0.14	1.61 0.18	3.21 0.32	1289 242	593 70	605 73	1198 140
400	35	534 39	1.62 0.10	1.62 0.10	3.24 0.19	1282 247	616 51	624 58	1240 99

Values are mean and standard deviation
Body weight at necropsy

Table 24 Relative Organ Weights in Male P Rats (%)

Dose (mg/kg/day)	Number of animals	Testis (g)			Prostate (mg)	Epididymus (mg)		
		Left	Right	Total		Left	Right	Total
0	35	0.304 0.032	0.301 0.033	0.605 0.064	0.221 0.043	0.115 0.013	0.116 0.014	0.231 0.026
25	34	0.306 0.029	0.305 0.030	0.610 0.058	0.249 0.048	0.116 0.014	0.120 0.014	0.236 0.027
100	35	0.302 0.025	0.301 0.032	0.603 0.055	0.242 0.041	0.111 0.013	0.114 0.014	0.225 0.026
400	35	0.304 0.023	0.304 0.024	0.608 0.046	0.241 0.046	0.116 0.012	0.117 0.013	0.233 0.023

Values are mean and standard deviation

Table 25 Number of F_1 Pups (Within 24 Hours of Birth)

Dose (mg/kg/day)	Number of dams	Number of pups within 24 hours			Sex ratio (Male %)
		Live	Dead	Total	
0	13	15.1 1.8	0.1 0.3	15.2 1.7	47.3 14.1
25	11	14.1 1.6	0.1 0.3	14.2 1.6	54.2 17.4
100	12	14.7 2.4	0.0 0.0	14.7 2.4	52.2 14.0
400	12	14.3 1.8	0.3 0.7	14.7 2.0	47.8 11.7

Values are mean and standard deviation

Table 26 Viability and Weaning Indices of F_1 Rats

Dose (mg/kg/day)	Number of dams a/b	Number of live pups							Viability Index (%)	Weaning Index (%)
				4 (Culling)						
		At birth	1	Before	After	7	14	21 (days)		
0	13/13	(197)	(196)	(196)	(104)	(104)	(104)	(104)		
		15.2	15.1	15.1	8.0	8.0	8.0	8.0	99.4	100.0
		1.7	1.8	1.8	0.0	0.0	0.0	0.0	2.1	0.0
25	11/11	(156)	(153)	(152)	(88)	(88)	(88)	(87)		
		14.2	13.9	13.8	8.0	8.0	8.0	7.9	97.6	98.9
		1.6	1.4	1.5	0.0	0.0	0.0	0.3	4.3	3.8
100	12/12	(176)	(176)	(176)	(96)	(96)	(96)	(96)		
		14.7	14.7	14.7	8.0	8.0	8.0	8.0	100.0	100.0
		2.4	2.4	2.4	0.0	0.0	0.0	0.0	0.0	0.0
400	12/12	(176)	(169)	(169)	(96)	(96)	(95)	(95)		
		14.7	14.1	14.1	8.0	8.0	7.9	7.9	96.3	99.0
		2.0	1.8	1.8	0.0	0.0	0.3	0.3	6.1	3.6

a: Number of dams at 1 day of age
b: Number of dams at 4 days of age
(Parentheses) indicate the total number of live pups. Other values are mean and standard deviation.
Viability Index: (Number of live pups at 4 days of age/Number of live pups at birth) × 100(%)
Weaning Index: (Number of live pups at 21 days of age/Number of live pups at 4 days of age (after culling) × 100 (%)

Table 27 Body Weight of F_1 Pups

Sex	Dose (mg/kg/day)	Number of dams a/b	Body weight (g)					
				4 (Culling)				
			1	Before	After	7	14	21 (days)
Male	0	13/13	6.6 0.6	9.6 1.0	9.6 1.0	16.1 1.7	32.3 2.9	51.9 4.7
	25	11/11	7.0 0.3	9.9 0.6	10.0 0.7	16.3 1.0	32.6 1.8	52.2 2.5
	100	12/12	6.7 0.6	9.6 0.7	9.8 0.7	15.6 1.4	32.4 2.2	51.3 3.3
	400	12/12	7.0 0.4	9.7 1.0	9.8 0.9	15.6 1.3	31.6 2.1	51.1 3.0
Female	0	13/13	6.4 0.5	9.2 0.9	9.4 0.9	15.4 1.5	31.3 2.6	49.9 3.8
	25	11/11	6.7 0.3	9.5 0.7	9.7 0.7	15.7 1.1	31.7 1.7	50.3 2.5
	100	12/12	6.4 0.5	9.1 0.6	9.2 0.6	14.5 1.0	30.4 2.1	48.1 3.2
	400	12/12	6.6 0.3	9.2 0.9	9.5 0.9	14.8 1.3	30.3 2.3	48.3 3.4

Values are mean and standard deviation
a: Number of dams at the age of 1 day.
b: Number of dams at the age of 4 days.

Table 28 Behavioral Development of Male F_1 Pups

Dose (mg/kg/day)	Number of dams	Surface righting reflex (2 days of age) (%)	Gait on paws (14 days of age) (%)	Pinna reflex (18 days of age) (%)	Pain response (21 days of age) (%)	Pupillary reflex (21 days of age) (%)
0	13	100.0 (13/13)	98.1 a) 6.9	100.0 (13/13)	100.0 (13/13)	100.0 (13/13)
25	11	100.0 (11/11)	100.0 0.0	100.0 (11/11)	100.0 (11/11)	100.0 (11/11)
100	12	100.0 (12/12)	100.0 0.0	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)
400	12	100.0 (12/12)	100.0 0.0	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)

a): Values are mean and standard deviation
(n1/n2): n1: Number of dams with pups showing normal response or reflex
n2: Number of dams examined

Table 29 Behavioral Development of Female F_1 Pups

Dose (mg/kg/day)	Number of dams	Surface reighting reflex (2 days of age) (%)	Gait on paws (14 days of age) (%)	Pinna reflex (18 days of age) (%)	Pain response (21 days of age) (%)	Pupillary reflex (21 days of age) (%)
0	13	100.0 (13/13)	100.0 a) 0.0	100.0 (13/13)	100.0 (13/13)	100.0 (13/13)
25	11	100.0 (11/11)	100.0 0.0	100.0 (11/11)	100.0 (11/11)	100.0 (11/11)
100	12	100.0 (12/12)	100.0 0.0	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)
400	12	100.0 (12/12)	100.0 0.0	100.0 (12/12)	100.0 (12/12)	100.0 (12/12)

a): Values are mean and standard deviation
(n1/n2): n1 : Number of dams with pups showing normal response or reflex
n2 : Number of dams examined

Table 30 Physical Development of Male F_1 Pups

Dose (mg/kg/day)	Number of dams	Pinna detachment (3 days of age) (%)	Fur appearance (3 days of age) (%)	External auditory canal opening (12 days of age) (%)	Incisor eruption (10 days of age) (%)	Eye opening (14 days of age) (%)	Penis formation (days of age)
0	13	99.4	100.0	71.2	59.6	76.9	41.8
		2.1	0.0	36.6	34.7	25.9	1.4
25	11	100.0	100.0	86.8	68.6	86.8	41.6
		0.0	0.0	16.9	40.6	20.3	0.8
100	12	99.2	100.0	62.9	70.8	81.3	42.7
		2.9	0.0	33.1	38.2	24.1	0.6
400	12	100.0	100.0	85.4	68.8	77.1	42.2
		0.0	0.0	31.0	33.9	29.1	1.0

Values are mean and standard deviation

Table 31 Physical Development of Female F_1 Pups

Dose (mg/kg/day)	Number of dams	Pinna detachment (3 days of age) (%)	Fur appearance (3 days of age) (%)	External auditory canal opening (12 days of age) (%)	Incisor eruption (10 days of age) (%)	Eye opening (14 days of age) (%)	Vaginal opening (days of age)
0	13	100.0	98.1	69.2	53.8	88.5	31.8
		0.0	6.9	41.0	40.6	16.5	1.0
25	11	97.7	100.0	86.4	58.3	89.4	31.5
		7.5	0.0	20.5	40.5	23.0	1.2
100	12	100.0	100.0	78.5	59.7	85.4	31.9
		0.0	0.0	23.7	35.9	22.5	0.8
400	12	97.1	100.0	91.0	66.7	91.7	32.1
		6.8	0.0	13.5	30.8	16.3	1.4

Values are mean and standard deviation

Table 32 Clinical Signs in Male F_1 Rats

	Number of animals with abnormalities			
	0[a]	25	100	400
Clinical Signs	52[b]	42	49	48
Abnormal findings	0	0	0	0

a: Dose (mg/kg/day)
b: Number of animals examined

Table 33 Mortality in Male F_1 Rats

Dose (mg/kg/day)	Number of animals	Number of death		Mortality (%)
		Before mating	During mating period	
0	52	0	0	0
25	42	1	0	2.4
100	49	0	0	0
400	48	0	1	2.1

Table 34 Clinical Signs in Female F_1 Rats

	Number of animals with abnormalities			
	[a] 0	25	100	400
Clinical Signs	[b] 52	45	47	47
Hemophthalmia	0	0	0	1

a: Dose (mg/kg/day)
b: Number of animals examined

Table 35 Mortality in Female F_1 Rats

Dose (mg/kg/day)	Number of animals	Number of death		Mortality (%)
		Before mating	During mating period	
0	52	0	0	0
25	45	0	0	0
100	47	0	0	0
400	47	0	0	0

Table 36 Body Weight of Male F_1 Rats

Dose (mg/kg/day)	Number of animals	Body weight (g)								
		3	4	5	6	7	8	9	10	(weeks)
		22	28	35	42	49	56	63	70	(days)
0	13	58	95	153	215	274	332	377	413	
		4	6	12	16	20	23	26	31	
25	11	57	92	151	212	270	326	372	410	
		3	5	9	11	15	17	21	29	
100	12	56	92	150	207	269	326	375	415	
		4	6	8	9	12	16	18	21	
400	12	56	90	147	205	265	322	369	415	
		3	5	8	11	14	18	20	22	

Values are mean and standard deviation

Table 37 Body Weight of Female F_1 Rats

Dose (mg/kg/day)	Number of animals	Body weight (g)								
		3	4	5	6	7	8	9	10	(weeks)
		22	28	35	42	49	56	63	70	(days)
0	13	54	85	126	158	184	208	228	251	
		4	5	9	13	16	21	24	27	
25	11	55	88	130	164	193	218	241	258	
		3	4	7	12	15	18	23	27	
100	12	53	81	124	159	188	211	234	251	
		5	6	12	16	21	23	26	30	
400	12	53	83	124	157	185	210	231	250	
		5	6	10	15	18	20	25	24	

Values are mean and standard deviation

Table 38 Body Weight of Female F_1 Rats (During Pregnancy)

Dose (mg/kg/day)	Number of animals	Body weight (g)					
		0	1	4	7	14	(days)
0	12	263 32	269 33	289 33	303 33	343 35	
25	11	264 28	272 29	287 27	301 28	336 29	
100	11	258 31	264 33	280 34	293 34	330 38	
400	12	263 20	268 22	286 22	303 25	341 29	

Values are mean and standard deviation

Table 39 Food intake of Male F_1 Rats

Dose (mg/kg/day)	Number of animals	Food intake (g)								
		3 23	4 30	5 37	6 44	7 51	8 58	9 65	10 72	(weeks) (days)
0	13	8 1	18 3	24 3	27 2	30 3	31 3	31 4	31 3	
25	11	7 1	18 2	23 2	27 2	30 3	30 4	31 3	31 3	
100	12	7 1	17 2	23 2	27 3	29 2	32 3	33 3	31 3	
400	12	7 2	17 1	23 2	26 1	30 1	31 2	32 3	31 3	

Values are mean and standard deviation

Table 40 Food Intake of Female F_1 Rats

Dose (mg/kg/day)	Number of animals	Food intake (g)								
		3	4	5	6	7	8	9	10	(weeks)
		23	30	37	44	51	58	65	70	(days)
0	13	6	16	19	19	21	21	23	22	
		1	1	2	3	3	2	4	4	
25	11	7	16	20	19	20	22	24	23	
		1	1	2	2	5	2	4	4	
100	12	6	15	19	20	21	22	24	22	
		1	1	2	3	2	3	3	3	
400	12	6	15	16	19	19	21	22	22	
		1	3	2	3	2	3	3	3	

Values are mean and standard deviation

Table 41 Food Intake of Female F_1 Rats (During Pregnancy)

Dose (mg/kg/day)	Number of animals	Food intake (g)				
		1	4	7	14	(days)
0	12	22	26	28	28	
		3	3	4	4	
25	11	22	26	27	26	
		3	2	4	6	
100	11	22	25	27	26	
		4	4	3	4	
400	12	22	27	28	28	
		2	2	3	3	

Values are mean and standard deviation

Table 42 Open Field Test (Male)

Dose (mg/kg/day)	Number of animals	Latency (sec)			Grooming [a]			Sniffing [a]			Rearing [a]			Defecation [b]			Urination [b]			Ambulation [c]		
		Trial			Trial			Trial			Trial			Trial			Trial			Trial		
		1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
0	10	20.7	9.9	4.8	0.4	0.2	0.3	9.0	7.0	6.5	3.2	4.5	4.8	3.3	2.9	1.8	1.9	2.9	1.3	30.0	47.6	67.1
		19.4	5.2	2.1	0.7	0.4	0.5	2.7	1.2	2.5	2.8	2.2	2.5	1.8	2.1	1.8	1.4	1.2	1.6	12.0	14.0	10.9
25	10	18.2	13.3	9.0**	0.1	0.4	0.2	8.0	4.8*	6.3	2.6	2.9	3.8	2.0	1.8	1.7	2.1	1.8	1.0	29.8	36.5	48.2
		7.3	11.8	4.0	0.3	0.7	0.4	2.1	0.9	1.9	2.9	2.5	2.8	1.8	1.5	2.0	1.4	2.0	1.2	16.3	24.6	17.4
100	10	12.8	7.9	4.6	0.1	0.5	0.3	10.2	5.6	4.8	3.9	6.3	6.1	2.1	1.5	1.8	1.5	0.9	0.7	34.8	52.2	58.0
		5.0	3.1	1.8	0.3	1.0	0.7	1.7	2.5	2.3	3.0	3.2	2.9	1.2	1.6	2.0	1.4	1.7	1.1	15.8	25.4	24.4
400	10	16.5	7.9	6.1	0	0.5	0.3	8.9	6.9	6.5	3.4	4.6	4.0	1.4	1.4	1.1	0.9	1.6	0.8	33.0	42.5	46.4
		6.0	2.0	2.3	0	0.8	0.7	2.5	2.1	2.1	2.0	4.5	2.6	2.0	1.9	1.7	0.6	1.7	1.2	18.8	15.6	30.8

a: Number of activities
b: Number of defecations or urinations
c: Number of squares traversed
Values are mean and standard deviation
* : P<0.05
** : P<0.01

Table 43 Open Field Test (Female)

Dose (mg/kg/day)	Number of animals	Latency (sec)			Grooming [a]			Sniffing [a]			Rearing [a]			Defecation [b]			Urination [b]			Ambulation [c]		
		Trial			Trial			Trial			Trial			Trial			Trial			Trial		
		1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
0	10	16.1	10.0	5.9	0.4	0.1	0.2	5.6	3.3	1.2	3.8	4.1	3.8	1.6	1.1	1.6	5.9	2.5	1.7	47.7	50.3	61.3
		17.8	7.5	3.2	0.5	0.3	0.6	2.5	2.1	1.4	3.1	2.6	1.7	1.9	1.2	1.5	3.6	2.3	1.8	21.9	17.3	24.9
25	10	12.2	15.5	7.4	0.3	0.4	0.2	4.3	3.1	1.6	4.4	3.6	6.8*	1.3	1.5	1.8	1.5*	3.4	2.2	50.0	51.3	76.3
		5.4	18.6	5.3	0.7	0.5	0.4	1.8	1.9	2.0	2.8	2.8	3.4	1.9	1.9	1.8	1.8	2.6	1.9	12.7	24.0	16.3
100	10	13.0	7.4	6.0	0.6	0.6	0.4	3.8	4.1	2.8	5.2	3.4	3.4	0.7	0.7	1.1	4.7	2.7	0.5	47.1	47.0	61.8
		11.1	3.7	1.9	0.7	0.5	0.7	2.0	1.7	1.0	2.9	2.1	1.9	0.8	1.2	1.4	4.6	3.1	0.5	14.0	19.3	31.8
400	10	10.0	4.6	4.8	0.6	0.4	0.4	3.1*	3.3	2.4	4.3	4.2	3.3	0.6	1.9	1.3	2.5	4.1	1.4	61.8	63.2	75.6
		6.4	1.5	2.6	0.8	0.7	0.5	1.2	1.9	1.6	2.4	2.6	2.1	1.0	2.5	1.4	3.0	3.6	1.3	7.5	27.6	20.6

a: Number of activities
b: Number of defecations or urinations
c: Number of squares traversed
Values are mean and standard deviation
* : P<0.05
** : P<0.01

Table 44 Swimming Time in the Water Multiple T-maze Test (Male)

Dose (mg/kg/day)	Number of animals	Swimming time at trial (1st day) (sec)					Swimming time at trial (2nd day) (sec)					Swimming time at trial (3rd day) (sec)				
		1	2	3	4	Mean	1	2	3	4	Mean	1	2	3	4	Mean
0	10	56.4	38.5	38.2	20.0	38.4	34.5	17.0	16.8	16.9	21.3	26.3	19.2	17.0	14.4	19.2
		31.7	17.2	21.8	8.7	9.2	20.3	4.7	4.9	6.1	5.9	18.2	5.8	4.9	3.4	5.2
25	10	67.6	63.9	35.5	33.0	50.0	41.9	30.8	25.3	20.9	29.7	32.3	21.6	21.1	18.0	23.3
		45.6	30.0	18.3	24.1	19.5	15.7	11.9	14.3	12.0	10.5	15.3	11.4	12.1	11.7	10.6
100	10	90.7	54.5	46.1	35.6	55.7	47.1	20.8	19.9	17.6	26.4	30.5	19.6	17.4	15.8	20.8
		46.0	32.1	31.4	19.2	19.1	45.7	8.2	8.7	6.7	11.9	19.4	7.0	6.2	4.2	7.8
400	10	72.9	55.0	36.7	26.6	47.8	33.2	20.6	17.7	21.8	23.4	21.7	21.0	19.1	19.2	20.2
		29.4	44.7	23.7	11.6	14.8	19.5	16.3	6.9	10.2	9.7	13.7	9.1	10.2	6.0	5.9

Values are mean and standard deviation

Table 45 Number of Errors in the Water Multiple T-maze Test (Male)

Dose (mg/kg/day)	Number of animals	Number of errors at trial (1st day)					Number of errors at trial (2nd day)					Number of errors at trial (3rd day)				
		1	2	3	4	Mean	1	2	3	4	Mean	1	2	3	4	Mean
0	10	3.7	2.1	2.2	0.4	2.1	2.2	0.3	0.2	0.3	0.8	1.3	0.4	0.2	0.2	0.5
		2.6	1.3	2.3	0.7	0.7	2.2	0.5	0.4	0.7	0.6	2.2	1.3	0.6	0.4	0.9
25	10	4.0	4.8	2.6	2.0	3.4	1.9	1.4	0.4	0.2	1.0	1.4	0.6	0.1	0	0.5
		3.5	3.3	2.3	2.6	2.0	1.7	1.2	0.7	0.4	0.8	1.7	0.8	0.3	0	0.5
100	10	6.2	3.2	3.0	2.3	3.6	2.2	0.5	0.6	0.2	0.9	1.2	0.4	0.3	0	0.5
		4.5	3.0	1.8	2.1	1.9	2.7	1.3	1.1	0.4	0.6	1.4	0.7	0.7	0	0.4
400	10	3.2	3.3	2.6	1.3	2.7	1.5	0.9	0.2	1.1	1.0	0.7	0.6	0.1	0.5	0.5
		2.0	2.5	2.3	1.1	0.8	1.7	1.5	0.4	1.7	1.0	1.3	0.8	0.3	0.7	0.4

Values are mean and standard deviation

Table 46 Swimming Time in the Water Multiple T-maze Test (Female)

Dose (mg/kg/day)	Number of animals	Swimming time at trial (1st day) (sec)					Swimming time at trial (2nd day) (sec)					Swimming time at trial (3rd day) (sec)				
		1	2	3	4	Mean	1	2	3	4	Mean	1	2	3	4	Mean
0	10	57.7	38.8	61.1	40.4	50.4	40.9	29.7	23.8	17.4	28.0	25.8	17.2	22.0	22.5	21.9
		23.0	24.1	53.8	38.5	23.9	47.7	17.9	13.4	7.7	17.6	20.1	6.8	24.0	25.0	14.4
25	10	51.5	68.5	36.4	36.6	47.8	34.4	23.2	20.4	18.8	24.2	21.3	16.1	18.6	18.0	18.5
		26.9	49.8	20.0	22.6	23.4	21.5	10.9	7.4	8.3	9.9	9.2	5.9	10.5	9.4	6.4
100	10	74.5	73.9	29.9	27.4	51.1	40.3	19.5	19.1	14.4	23.3	26.3	15.3	13.8	13.8	17.3
		40.7	57.1	12.9	23.8	18.1	28.3	6.5	9.8	4.4	8.1	12.5	5.6	4.4	4.9	4.7
400	10	68.5	47.6	34.8	34.3	46.3	25.0	18.5	16.1	17.2	19.2	15.4	15.0	14.3	14.7	14.9
		35.6	30.7	10.7	20.2	14.3	10.9	7.8	6.7	7.2	5.7	6.7	5.2	6.2	3.5	3.9

Values are mean and standard deviation

Table 47 Number of Errors in the Water Multiple T-maze Test (Female)

Dose (mg/kg/day)	Number of animals	Number of errors at trial (1st day)					Number of errors at trial (2nd day)					Number of errors at trial (3rd day)				
		1	2	3	4	Mean	1	2	3	4	Mean	1	2	3	4	Mean
0	10	4.2	2.3	4.7	3.7	3.7	3.0	2.6	1.7	0.7	2.0	2.1	0.5	0.4	0.3	0.9
		2.8	1.5	4.3	4.8	2.4	4.6	2.5	1.6	1.1	1.9	2.3	0.7	1.3	0.7	0.7
25	10	2.9	5.3	2.6	2.8	3.4	2.1	1.5	0.9	0.5	1.3	1.5	0.5	0.8	0.3	0.8
		1.7	5.3	2.0	2.4	2.2	1.5	1.2	0.9	0.7	0.7	1.8	0.8	1.0	0.7	0.6
100	10	5.6	5.8	1.9	1.4	3.6	2.8	1.5	0.9	0.2	1.4	2.0	0.3	0.1	0.1	0.7
		3.6	5.2	1.7	2.0	1.9	2.7	2.3	1.6	0.6	1.0	2.0	0.5	0.3	0.3	0.5
400	10	4.1	3.5	2.4	2.5	3.2	1.3	1.0	0.7	0.9	1.0	0.4	0.4	0.3	0.3	0.4
		2.6	2.4	1.1	2.5	1.3	1.3	1.2	1.3	1.1	0.8	0.7	0.7	0.7	0.5	0.3

Values are mean and standard deviation

Table 48 Copulation and Fertility Indices of Male F_1 Rats

Dose (mg/kg/day)	Number of males mated	Number of males copulated successfully	Copulation Index (%)	Number of fertile males	Fertility Index (%)
0	13	13	100	12	92.3
25	11	11	100	11	100
100	12	12	100	11	91.7
400	12	12	100	12	100

Copulation index = (number of males copulating successfully/number of males mated) × 100 (%)
Fertility index = (number of males having fertilized females/number of males copulating successfully) × 100 (%)

Table 49 Copulation and Fertility Indices of Female F_1 Rats

Dose (mg/kg/day)	Number of females mated	Number of females copulated successfully	Copulation Index (%)	Number of pregnant females	Fertility Index (%)
0	13	13	100	12	92.3
25	11	11	100	11	100
100	12	12	100	11	91.7
400	12	12	100	12	100

Copulation index = (number of females copulating successfully/number of females mated) × 100 (%)
Fertility index = (number of pregnant females/number of females copulating successfully) × 100 (%)

Table 50 Ovulation, Implantation and Embryo Survival at Day 14 of Pregnancy of F_1 Rats

Dose (mg/kg/day)	Number of Animals	Number of corpora lutea	Number of implants	Implantation rate (%)	Number of dead embryos	Embryo lethality rate (%)	Number of live embryos	Survival rate of embryos (%)
		(230)	(182)		(16)		(166)	
0	12	19.2	15.2	82.0	1.3	8.3	13.8	91.7
		3.3	2.6	20.3	1.4	8.2	2.2	8.2
		(199)	(165)		(8)		(157)	
25	11	18.1	15.0	84.1	0.7	4.6	14.3	95.4
		2.9	1.8	11.6	0.8	4.9	1.6	4.9
		(196)	(170)		(8)		(162)	
100	11	17.8	15.5	88.7	0.7	4.8	14.7	95.2
		3.5	1.6	12.9	0.6	4.3	1.9	4.3
		(219)	(194)		(7)		(187)	
400	12	18.3	16.2	89.7	0.6	3.7	15.6	96.3
		2.7	1.9	11.8	0.8	4.8	2.1	4.8

Values are mean and standard deviation
(Parentheses) indicate a total

Table 51 Gross Findings in Male F_1 Rats at 7 Weeks of Age

	Number of animals with abnormalities			
	[a] 0	25	100	400
Findings	[b] 26	20	25	24
Abnormal findings	0	0	0	0

a: Dose (mg/kg/day)
b: Number of animals examined

Table 52 Gross Findings in Female F_1 Rats at 7 Weeks of Age

	Number of animals with abnormalities			
	[a] 0	25	100	400
Findings	[b] 26	23	23	23
Abnormal findings	0	0	0	0

a: Dose (mg/kg/day)
b: Number of animals examined

Table 53 Absolute Organ Weights in Male F_1 Rats at 7 Weeks of Age

Dose (mg/kg/day)	Number of animals	Body weight (g)	Liver (g)	Spleen (mg)	Kidney			Heart (g)	Lung (g)	Adrenal gland			Thymus (mg)	Pituitary (mg)
					Left	Right	Total (g)			Left	Right	Total (mg)		
0	13	302	14.0	814	1.22	1.23	2.44	1.06	1.18	28.4	25.5	53.9	707	11.2
		35	1.9	132	0.16	0.18	0.34	0.11	0.08	4.8	4.2	8.6	91	1.8
25	10	314	14.4	767	1.31	1.34	2.64	1.21**	1.22	29.9	29.6	59.5	679	12.0
		14	0.9	117	0.08	0.11	0.18	0.14	0.10	5.2	6.0	10.4	127	1.5
100	12	305	14.3	782	1.27	1.28	2.55	1.11	1.23	29.1	29.6	58.6	725	11.0
		24	1.9	87	0.12	0.11	0.22	0.10	0.05	4.6	4.8	7.3	113	2.2
400	12	310	14.3	819	1.24	1.28	2.52	1.10	1.25	30.4	29.0	59.5	706	11.7
		17	0.9	97	0.08	0.09	0.16	0.07	0.07	3.5	3.0	5.9	182	1.9

Testis			Prostate (mg)	Brain (g)
Left	Right	Total (g)		
1.34	1.32	2.66	463	1.96
0.11	0.08	0.17	84	0.08
1.33	1.36	2.69	510	1.97
0.06	0.09	0.15	162	0.05
1.34	1.35	2.68	428	1.98
0.12	0.12	0.23	144	0.06
1.36	1.36	2.72	399	1.98
0.04	0.06	0.10	100	0.08

Values are mean and standard deviation
Body weight: at necropsy
**: P<0.01

Table 54 Relative Organ Weights in Male F_1 Rats at 7 Weeks of Age

(%)

Dose (mg/kg/day)	Number of animals	Liver	Spleen	Kidney			Heart	Lung	Adrenal gland			Thymus	Pituitary
				Left	Right	Total			Left	Right	Total		
0	13	4.62	0.272	0.403	0.407	0.810	0.353	0.395	0.0094	0.0084	0.0178	0.238	0.0037
		0.25	0.057	0.032	0.042	0.071	0.017	0.035	0.0012	0.0011	0.0021	0.046	0.0005
25	10	4.57	0.244	0.416	0.425	0.842	0.384**	0.390	0.0096	0.0095	0.0191	0.216	0.0038
		0.16	0.037	0.027	0.029	0.053	0.034	0.028	0.0020	0.0023	0.0041	0.038	0.0005
100	12	4.67	0.256	0.417	0.420	0.837	0.364	0.404	0.0095	0.0097	0.0192	0.238	0.0036
		0.37	0.022	0.023	0.029	0.049	0.025	0.032	0.0011	0.0015	0.0019	0.036	0.0006
400	12	4.63	0.264	0.400	0.415	0.814	0.354	0.405	0.0098	0.0094	0.0192	0.227	0.0038
		0.17	0.023	0.024	0.030	0.051	0.016	0.021	0.0010	0.0011	0.0019	0.055	0.0006

Testis			Prostate	Brain
Left	Right	Total		
0.447	0.441	0.888	0.154	0.656
0.040	0.046	0.082	0.026	0.069
0.425	0.434	0.859	0.162	0.627
0.033	0.038	0.069	0.049	0.030
0.440	0.445	0.885	0.141	0.650
0.055	0.054	0.108	0.048	0.044
0.440	0.439	0.879	0.129	0.641
0.024	0.029	0.052	0.030	0.039

Values are mean and standard deviation

**: P<0.01

Table 55 Absolute Organ Weight in Female F_1 Rats at 7 Weeks of Age

Dose (mg/kg/day)	Number of animals	Body weight (g)	Liver (g)	Spleen (mg)	Kidney			Heart (g)	Lung (g)	Adrenal gland			Thymus (mg)	Pituitary (mg)
					Left	Right	Total (g)			Left	Right	Total (mg)		
0	13	207	9.5	548	0.91	0.93	1.83	0.82	1.00	37.7	34.4	72.1	581	11.7
		17	1.2	54	0.09	0.08	0.16	0.08	0.08	6.4	5.5	11.2	106	2.2
25	11	202	9.2	499	0.94	0.94	1.88	0.83	0.97	36.7	36.3	73.1	529	11.5
		18	1.2	106	0.10	0.10	0.19	0.08	0.08	4.3	3.8	7.3	136	1.9
100	12	207	9.5	529	0.92	0.92	1.85	0.81	1.02	34.3	35.7	70.0	603	13.2
		15	1.0	71	0.09	0.09	0.18	0.06	0.10	7.3	5.1	10.5	91	1.7
400	12	209	9.2	538	0.92	0.93	1.85	0.83	1.00	36.4	35.9	72.3	656	12.5
		16	0.8	63	0.05	0.06	0.10	0.07	0.06	7.3	7.0	11.4	222	2.5

Ovary			Uterus (mg)	Brain (g)
Left	Right	Total (g)		
52.5	44.8	97.3	594	1.89
6.1	6.8	10.3	201	0.09
48.9	51.2	100.2	499	1.87
13.5	12.8	23.0	172	0.06
46.7	50.3	97.0	510	1.88
5.5	8.7	10.2	182	0.06
42.3*	45.0	87.3	502	1.87
6.5	7.0	12.2	156	0.08

Values are mean and standard deviation
Body weight: at necropsy
*: $P<0.05$

Table 56 Relative Organ Weights in Female F_1 Rats at 7 Weeks of Age (%)

Dose (mg/kg/day)	Number of animals	Liver	Spleen	Kidney			Heart	Lung	Adrenal gland			Thymus	Pituitary
				Left	Right	Total			Left	Right	Total		
0	13	4.58	0.266	0.439	0.448	0.887	0.396	0.484	0.0182	0.0166	0.0348	0.281	0.0057
		0.29	0.026	0.032	0.028	0.058	0.024	0.038	0.0024	0.0022	0.0042	0.043	0.0010
25	11	4.52	0.245	0.467	0.467	0.934	0.413	0.482	0.0183	0.0180	0.0363	0.262	0.0058
		0.26	0.040	0.044	0.037	0.077	0.026	0.037	0.0026	0.0018	0.0041	0.063	0.0012
100	12	4.59	0.256	0.446	0.448	0.894	0.391	0.496	0.0166	0.0173	0.0340	0.293	0.0064
		0.22	0.022	0.033	0.031	0.062	0.020	0.030	0.0034	0.0024	0.0048	0.046	0.0008
400	12	4.42	0.258	0.441	0.448	0.889	0.396	0.481	0.0174	0.0172	0.0346	0.312	0.0060
		0.27	0.025	0.030	0.037	0.064	0.016	0.040	0.0034	0.0033	0.0050	0.086	0.0013

Ovary			Uterus	Brain
Left	Right	Total		
0.0255	0.0219	0.0474	0.286	0.917
0.0032	0.0044	0.0068	0.091	0.068
0.0242	0.0252	0.0494	0.249	0.930
0.0061	0.0053	0.0096	0.086	0.082
0.0227	0.0244	0.0471	0.249	0.916
0.0027	0.0040	0.0048	0.094	0.079
0.0204**	0.0217	0.0421	0.242	0.899
0.0035	0.0038	0.0068	0.080	0.065

Values are mean and standard deviation
**: P<0.01

Table 57 Gross Findings in Male F_1 Rats at 10 Weeks of Age

	Number of animals with abnormalities			
	[a] 0	25	100	400
Findings	[b] 13	10	12	12
Abnormal findings	0	0	0	0

a: Dose (mg/kg/day)
b: Number of animals examined

Table 58 Gross Findings in Female F_1 Rats at 10 Weeks of Age

	Number of animals with abnormalities			
	[a] 0	25	100	400
Findings	[b] 13	11	12	12
Hemophthalmia (Unilateral)	0	0	0	1

a: Dose (mg/kg/day)
b: Number of animals examined

Table 59 Absolute Organ Weights in Male F_1 Rats at 10 Weeks of Age

Dose (mg/kg/day)	Number of animals	Body weight (g)	Liver (g)	Spleen (mg)	Kidney			Heart (g)	Lung (g)	Adrenal gland			Thymus (mg)	Pituitary (mg)
					Left	Right	Total (g)			Left	Right	Total (mg)		
0	13	435	17.2	764	1.50	1.51	3.01	1.36	1.32	31.4	30.9	62.3	609	12.0
		34	2.2	95	0.15	0.14	0.29	0.15	0.08	5.8	6.1	11.1	110	1.4
25	10	428	16.7	797	1.52	1.53	3.05	1.31	1.34	31.6	28.9	60.4	684	11.9
		32	1.6	150	0.10	0.09	0.19	0.09	0.10	4.9	3.8	7.6	161	1.7
100	12	438	17.6	882	1.57	1.55	3.12	1.31	1.32	33.2	31.2	64.4	705	13.0
		21	1.2	190	0.10	0.11	0.20	0.09	0.09	3.4	5.4	7.6	119	1.7
400	12	442	18.0	835	1.57	1.57	3.14	1.33	1.39	34.7	31.5	66.2	696	13.2
		25	1.9	143	0.14	0.14	0.27	0.09	0.11	5.7	5.0	10.4	114	0.6

Testis			Prostate (mg)	Brain (g)
Left	Right	Total (g)		
1.55	1.56	3.11	798	2.10
0.07	0.08	0.14	82	0.05
1.58	1.58	3.17	787	2.04
0.12	0.14	0.26	135	0.10
1.55	1.55	3.10	710	2.07
0.12	0.10	0.21	102	0.08
1.58	1.60	3.18	743	2.08
0.10	0.11	0.21	149	0.05

Values are mean and standard deviation
Body weight: at necropsy

Table 60 Relative Organ Weights in Male F_1 Rats at 10 Weeks of Age

Dose (mg/kg/day)	Number of animals	Liver	Spleen	Kidney			Heart	Lung	Adrenal gland			Thymus	Pituitary
				Left	Right	Total			Left	Right	Total		
0	13	3.95	0.176	0.344	0.348	0.692	0.313	0.304	0.0072	0.0071	0.0143	0.140	0.0028
		0.25	0.018	0.020	0.020	0.039	0.021	0.017	0.0012	0.0012	0.0023	0.021	0.0003
25	10	3.90	0.186	0.355	0.360	0.715	0.308	0.314	0.0074	0.0068	0.0141	0.160	0.0028
		0.19	0.027	0.022	0.027	0.048	0.020	0.026	0.0009	0.0009	0.0016	0.039	0.0004
100	12	4.03	0.201	0.360	0.354	0.714	0.300	0.303	0.0076	0.0071	0.0147	0.161	0.0030
		0.18	0.042	0.024	0.021	0.043	0021	0.020	0.0008	0.0012	0.0018	0.027	0.0004
400	12	4.08	0.189	0.355	0.357	0.713	0.302	0.315	0.0079	0.0071	0.0150	0.158	0.0030
		0.33	0.031	0.034	0.033	0.066	0.023	0.029	0.0013	0.0012	0.0024	0.027	0.0003

Testis			Prostate	Brain
Left	Right	Total		
0.360	0.360	0.720	0.184	0.486
0.034	0.035	0.069	0.021	0.039
0.372	0.372	0.744	0.185	0.480
0.043	0.044	0.087	0.033	0.038
0.355	0.354	0.710	0.163	0.473
0.025	0.021	0.045	0.027	0.023
0.360	0.364	0.724	0.168	0.472
0.037	0.041	0.078	0.029	0.029

Values are mean and standard deviation

Table 61 Absolute Organ Weights in Female F_1 Rats at 10 Weeks of Age

Dose (mg/kg/day)	Number of animals	Body weight (g)	Liver (g)	Spleen (mg)	Kidney			Heart (g)	Lung (g)	Adrenal gland			Thymus (mg)	Pituitary (mg)
					Left	Right	Total (g)			Left	Right	Total (mg)		
0	13	256	9.7	552	0.95	0.97	1.92	0.91	1.04	40.5	37.1	77.6	504	12.5
		31	1.5	93	0.08	0.09	0.17	0.08	0.07	5.4	7.5	11.7	106	1.3
25	11	270	10.2	562	1.01	1.00	2.01	0.93	1.05	39.9	34.5	74.3	539	12.3
		30	1.7	162	0.11	0.11	0.22	0.13	0.14	9.2	7.3	15.3	122	2.7
100	12	260	9.9	572	1.00	1.01	2.02	0.93	1.02	38.8	36.6	75.4	560	12.9
		31	1.3	85	0.12	0.10	0.21	0.18	0.11	7.5	4.9	11.1	121	1.3
400	12	257	9.1	549	0.98	0.99	1.97	0.89	1.03	40.3	37.0	77.3	529	12.0
		26	1.0	97	0.10	0.10	0.20	0.09	0.06	5.5	4.3	9.5	148	1.5

Ovary			Uterus (mg)	Brain (g)
Left	Right	Total (mg)		
58.0	56.7	114.7	572	1.93
12.8	9.8	20.0	168	0.07
56.9	59.5	116.4	543	1.90
10.0	13.7	21.0	228	0.10
51.3	55.8	107.1	587	1.91
11.1	8.7	17.5	180	0.07
46.6*	53.5	100.1	541	1.92
4.0	10.6	12.5	160	0.11

Values are mean and standard deviation
Body weight: at necropsy
*: $P<0.05$

Table 62 Relative Organ Weights in Female F_1 Rats at 10 Weeks of Age (%)

Dose (mg/kg/day)	Number of animals	Liver	Spleen	Kidney			Heart	Lung	Adrenal gland			Thymus	Pituitary
				Left	Right	Total			Left	Right	Total		
0	13	3.78	0.215	0.374	0.362	0.755	0.356	0.408	0.0159	0.0145	0.0304	0.196	0.0049
		0.26	0.021	0.032	0.033	0.063	0.025	0.035	0.0022	0.0024	0.0040	0.030	0.0006
25	11	3.75	0.205	0.374	0.373	0.747	0.346	0.390	0.0148	0.0127	0.0275	0.199	0.0045
		0.30	0.039	0.026	0.024	0.048	0.021	0.030	0.0032	0.0022	0.0048	0.034	0.0006
100	12	3.79	0.221	0.387	0.392	0.779	0.357	0.399	0.0149	0.0143	0.0292	0.215	0.0050
		0.14	0.028	0.029	0.045	0.070	0.052	0.065	0.0025	0.0026	0.0044	0.038	0.0007
400	12	3.55	0.213	0.384	0.364	0.768	0.346	0.401	0.0157	0.0145	0.0301	0.205	0.0047
		0.26	0.030	0.035	0.031	0.064	0.032	0.032	0.0016	0.0014	0.0029	0.043	0.0006

Ovary			Uterus	Brain
Left	Right	Total		
0.0226	0.0222	0.0448	0.223	0.761
0.0040	0.0032	0.0060	0.060	0.083
0.0212	0.0219	0.0431	0.203	0.709
0.0037	0.0037	0.0059	0.084	0.066
0.0198	0.0218	0.0416	0.227	0.743
0.0040	0.0044	0.0075	0.070	0.088
0.0182**	0.0209	0.0392	0.212	0.752
0.0020	0.0045	0.0057	0.063	0.051

Values are mean and standard deviation
**: P<0.01

Table 63 Gross Findings in Male F_1 Rats after Mating

	Number of animals with abnormalities			
	0[a]	25	100	400
Findings	13[b]	12	12	12
Appearance				
Cyanoses of limbs	0	0	0	1[d]
Liver				
Congestion	0	1[c]	0	1[d]
Cloudy	0	0	0	1[d]
Lungs				
Congestion	0	1[c]	0	1[d]
Thymus				
Patchy hemorrhage	0	0	0	1[d]
Testes				
Bilateral atrophy	0	0	1	0
Unilateral swelling	0	0	0	1

a: Dose (mg/kg/day)
b: Number of animals examined
c: Died at 10 weeks of age
d: Died at 11 weeks of age

Table 64 Postmortem Examination of Female F_1 Rats on Day 14 of Pregnancy

	Number of animals with abnormalities			
	0[a]	25	100	400
Findings	12[b]	11	11	12
Abnormal findings	0	0	0	0

a: Dose (mg/kg/day)
b: Number of animals examined

Table 65 Absolute Organ Weights in Male F_1 Rats Used for Evaluation of Reproductive Function

Dose (mg/kg/day)	Number of animals	Body weight (g)	Testis			(g)	Prostate (mg)	Epididymis			(mg)
			Left	Right	Total			Left	Right	Total	
0	13	453	1.61	1.60	3.20		806	575	569	1144	
		42	0.11	0.09	0.19		202	40	37	73	
25	11	465	1.64	1.65	3.29		911	601	601	1202	
		37	0.11	0.11	0.21		190	53	47	95	
100	12	454	1.56	1.54	3.09		839	549	566	1115	
		38	0.24	0.32	0.56		164	80	69	144	
400	11	472	1.70	1.59	3.29		770	546	571	1117	
		29	0.50	0.09	0.55		176	47	46	86	

Values are mean and standard deviation
Body weight: at necropsy

Table 66 Relative Organ Weights in Male F_1 Rats Used for Evaluation of Reproductive Function (%)

Dose (mg/kg/day)	Number of animals	Testis			Prostate	Epididymis		
		Left	Right	Total		Left	Right	Total
0	13	0.357	0.355	0.712	0.179	0.128	0.126	0.254
		0.037	0.030	0.066	0.048	0.013	0.010	0.023
25	11	0.355	0.357	0.712	0.198	0.130	0.130	0.259
		0.041	0.043	0.083	0.048	0.008	0.005	0.012
100	12	0.341	0.335	0.676	0.185	0.121	0.125	0.245
		0.042	0.061	0.102	0.036	0.014	0.012	0.024
400	11	0.361	0.338	0.699	0.163	0.116	0.121	0.237
		0.110	0.031	0.128	0.038	0.013	0.014	0.025

Values are mean and standard deviation

Skin Photosensitization Test of Arbutin in Guinea Pigs

Skin Photosensitization Test of Arbutin in Guinea Pigs

Yoshio Katsumura, Mariko Uchiyama,
and Toshiaki Kobayashi

1. Introduction

This study evaluated the photosensitization potential of Arbutin in guinea pigs.

The study was conducted between March 10 and April 2, 1986.

2. Materials and Methods

2.1 Test substance

Arbutin (Lot a, Nippon Fine Chemical Co., Ltd.) was used as the test substance.

Since the original substance is a crystalline powder, it was dissolved in 50%, v/v, aqueous ethanol to a 10% concentration for application.

The positive control substance was 6-methylcoumarin (6-MC, Lot No. AHCL, Tokyo Kasei Kogyo Co., Ltd.) dissolved in ethanol to 1.0% and 0.1% concentrations for application.

2.2 Animals

Hartley strain female albino guinea pigs weighing about 350 g were purchased. After an acclimation period of one week, animals weighing between 380 and 450 g that appeared normal were used.

2.3 Environmental conditions

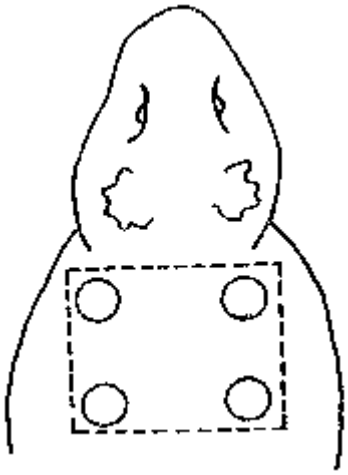
Animals were housed individually in aluminum guinea pig bracket cages (260 x 170 x 380 mm: CLEA Japan, Inc., Tokyo, Japan). They were fed laboratory chow (RC-4: Oriental Yeast Co., Ltd.) and tap water *ad libitum*. Animal quarters were automatically controlled to 23 ± 2ºC and 55 ± 5% relative humidity.

2.4 Skin photosensitization test method

The adjuvant-and-strip method[1),2)] was used.

2.4.1 Photosensitization induction method

Twenty-five guinea pigs were used; ten were treated with Arbutin, five with 6-MC, and ten with distilled water (control group). Fur in the shoulder area was clipped with electric clippers and then shaved with an electric shaver. The following operations were performed on the 3 × 4 cm area from which fur was removed:



1) Freund's complete adjuvant (Difco Laboratories) and the same quantity of sterile water (Otsuka Pharmaceutical Co., Ltd.) were emulsified (water-in-oil type emulsion), and 0.1 mL of this emulsion was injected intradermally into the four points marked with a circle in the figure.
2) The stratum corneum was abraded on the neck at the site of the intradermal injection using adhesive tape.
3) One-hundred microliters of 10% Arbutin in 50%, v/v, aqueous ethanol was applied to the stripped section in the photosensitization group, and 0.1 mL of 5% 6-MC ethanol solution was applied to the 6-MC group. Distilled water was applied to the control group.
4) After application, the area was irradiated with UVA at 10.2 joules/cm². The light source consisted of six black light fluorescent tubes (Toshiba FL40S BLB, λ = 300 to 400 nm, λmax = 360 nm) equipped with a glass filter to eliminate radiation below 320 nm. The distance from the light source to the skin was 10 cm.
5) Steps 2) to 4) were repeated once daily for 5 consecutive days.

2.4.2 Photosensitization challenge method

Challenge was carried out on Day 21 after the initial sensitization. Fur was removed from the flank by clipping and shaving as before. Guinea pigs were restrained in the prone position. Aliquots (20 μl) of a 10% solution of Arbutin in 50%, v/v, aqueous ethanol were applied in pairs to 1.5 x 1.5 cm areas of skin on both the left- and right-hand sides of the midline to the backs of animals photosensitized with arbutin; 1 and 0.1% 6-MC ethanol solution was applied to the backs of animals photosensitized with 6-MC. The test substance was similarly applied to animals in the control group. One side was irradiated with UVA at 10.2 joules/cm² while the other side served as a control and was covered with aluminum foil to prevent exposure to light.

Skin reactions were evaluated with respect to erythema and edema at 24 and 48 hours after challenge exposure.

Criteria

(1) Erythema formation

Criteria	Score
No Erythema	0
Very slight erythema	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema with defined scar formation	4

(2) Edema formation

Criteria	Score
No edema	0
Slight edema	1
Moderate edema	2
Severe edema	3

3. Results

Table 1 shows the results of evaluation of skin photosensitization of Arbutin according to the adjuvant-and-strip method. Table 2 shows the results of evaluation of 6-MC used as the positive control substance.

No positive reaction was observed in animals in either the treated group or control group challenged with 10% Arbutin in 50%, v/v, aqueous ethanol.

Conversely, strong photosensitization reactions were observed in the 6-MC group.

Table-1 Results of skin photosensitization test of Arbutin

Site		Irradiated									Non-Irradiated								
Group	Hours after challenge exposure	Score									Score								
		Erythema					Edema				Erythema					Edema			
		0	1	2	3	4	0	1	2	3	0	1	2	3	4	0	1	2	3
Photosensitization	24	10	0	0	0	0	10	0	0	0	10	0	0	0	0	10	0	0	0
	48	10	0	0	0	0	10	0	0	0	10	0	0	0	0	10	0	0	0
Control	24	10	0	0	0	0	10	0	0	0	10	0	0	0	0	10	0	0	0
	48	10	0	0	0	0	10	0	0	0	10	0	0	0	0	10	0	0	0

(Note) Photosensitization induction substance: Arbutin, 10% in 50%, v/v, aqueous ethanol
Photosensitization challenge substance: Arbutin, 10% in 50%, v/v, aqueous ethanol

Control group: Treated the same as the photosensitization group using distilled water instead of the test substance.
For challenge, the test substance was injected intradermally using the same procedure as in the photosensitization induction group.

Table-2 Results of skin photosensitization test of 6-MC (Positive control substance)

Site			Irradiated									Non-irradiated								
Group	Challenge concentration	Hours after challenge exposure	Score									Score								
			Erythema					Edema				Erythema					Edema			
			0	1	2	3	4	0	1	2	3	0	1	2	3	4	0	1	2	3
Photo-sensitization	1%	24	0	1	2	2	0	4	1	0	0	5	0	0	0	0	5	0	0	0
		48	0	0	2	3	0	3	2	0	0	5	0	0	0	0	5	0	0	0
	0.1%	24	4	1	0	0	0	5	0	0	0	5	0	0	0	0	5	0	0	0
		48	3	1	1	0	0	5	0	0	0	5	0	0	0	0	5	0	0	0
Control	1%	24	10	0	0	0	0	10	0	0	0	10	0	0	0	0	10	0	0	0
		48	10	0	0	0	0	10	0	0	0	10	0	0	0	0	10	0	0	0
	0.1%	24	10	0	0	0	0	10	0	0	0	10	0	0	0	0	10	0	0	0
		48	10	0	0	0	0	10	0	0	0	10	0	0	0	0	10	0	0	0

(Note) Photosensitization induction substance: 6-MC, 5% in aqueous ethanol
Photosensitization challenge substance: 6-MC, 1 and 0.1% in aqueous ethanol

Control group: Treated the same as the photosensitization group using distilled water instead of the test substance.
For challenge, the test substance was injected intradermally using the same procedure as in the photosensitization induction group.

4. Conclusion

The photosensitization potential of Arbutin was evaluated in guinea pigs according to the adjuvant-and-strip method. No skin reactions were observed in animals in the photosensitization group. Consequently, it is concluded that Arbutin does not possess photosensitizing potential under the test conditions.

Conversely, strong photosensitization reactions were observed in the 6-MC group used for positive controls.

References

1) Yoshihisa Sato, Yoshio Katsumura, Hideyuki Ichikawa, Toshiaki Kobayashi, and Keisuke Nakajima: Photosensitization test method in guinea pigs, Nishi-Nippon Dermatology 42 (5), 831-837, 1980
2) Ichikawa H., Armstrong, R.B. and Harber, L.C.: Photoallergic contact dermatitis in guinea pigs. Improved induction technique using Freund's complete adjuvant. J. Invest. Dermatol. 76, 498-501, 1981

Phototoxicity Test of Arbutin in Guinea Pigs

Phototoxicity Test of Arbutin in Guinea Pigs

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1. Introduction

This study evaluated phototoxicity of Arbutin in guinea pigs.

The study was conducted from March 24 to March 28, 1986.

2. Materials and Methods

2.1 Test substance

Arbutin (Lot a, Nippon Fine Chemical Co., Ltd.) was used as the test substance.

Since the original substance is a crystalline powder, it was dissolved in 50%, v/v, aqueous ethanol to a 10% concentration for application.

8-Methoxypsoralen (Lot No. 61F-7702, Sigma Chemical Company) was used as the positive control substance. It was dissolved in ethanol to a 0.02% concentration.

2.2 Animals

Hartley strain male albino guinea pigs weighing about 380 g were purchased. After a one-week acclimatization, animals weighed 410 to 480 g and those appeared normal were used.

2.3 Environmental conditions

Animals were housed individually in aluminum guinea pig bracket cages (260 x 170 x 380 mm: CLEA Japan, Inc., Tokyo, Japan). They were fed laboratory chow (RC-4: Oriental Yeast Co., Ltd.) and tap water *ad libitum*. Animal quarters were automatically controlled to 23 ± 2ºC and 55 ± 5% relative humidity.

2.4 Phototoxicity test method

The Morikawa method[1)] was used. Fur on the back of 10 guinea pigs was clipped with an electric clipper and depilated with Shiseido hair remover. The test was carried out 24 hours after depilation.

2.4.1 Irradiation test

Aliquots (20 μl) of test substance were applied to two 1.5 × 1.5 cm areas of depilated skin, using the dorsal midline of the guinea pig as the axis of symmetry. Immediately after application, one side was covered with aluminum foil. Thirty minutes later, the other side was irradiated with six Toshiba model FL-40 BLB lamps (emission: 300-400 nm, λ_{max}=360 nm) arranged in parallel and fitted with a window-glass filter to eliminate radiation below 320 nm. The distance from the light source to the skin was 10 cm and the energy used was 14.0 joules/cm^2.

2.4.2 Evaluation

After irradiation, the guinea pigs were individually housed in an aluminum guinea pig bracket cage. Skin reactions with respect to erythema and edema were evaluated at 24, 48, and 72 hours after irradiation according to the following scoring criteria.

Criteria

a) List of scores

	Criteria	Score
Erythema	No erythema	0
	Very slight erythema	1
	Well-defined erythema	2
	Severe erythema	3
Edema	No edema	0
	Slight edema	1
	Severe edema	2

Phototoxicity was evaluated by comparing scores of the irradiated and non-irradiated sections. Differences were calculated for each observation time and averaged. After calculating the ratings, evaluation was made according to the following criteria:

b) Criteria of Phototoxicity

Rating	Evaluation
0 to 0.5	Almost no Phototoxicity
0.6 to 1.2	Minor Phototoxicity
1.3 to 2.5	Phototoxicity present
2.5 to 5.0	Strong Phototoxicity

$$\text{Phototoxicity rate} = \frac{\text{Number of positive animals}}{\text{Number of animals}}$$

$$\text{Mean response} = \frac{\text{Total scores for erythema and edema}}{\text{Number of animals}}$$

Rating = (Average of irradiated sections) - (Average of non-irradiated sections)

3. Results

Phototoxicity of a 10% solution of Arbutin and the positive control substance, 8-methoxypsoralen, were evaluated. The test results are shown in Table 1 (Parts 1 and 2).

No skin reactions were observed in either irradiated or non-irradiated sections treated with Arbutin. Conversely, a strong phototoxicity reaction was observed with the positive control substance, 8-methoxypsoralen.

Table -1 Results of Phototoxicity Test of Arbutin and 8-Methoxypsoralen

Part 1

Test/control substance	Hours after irradiation	Irradiated site							Non-irradiated site						
		Reaction score							Reaction score						
		Erythema				Edema			Erythema				Edema		
		0	1	2	3	0	1	2	0	1	2	3	0	1	2
Arbutin *	24	10	0	0	0	10	0	0	10	0	0	0	10	0	0
	48	10	0	0	0	10	0	0	10	0	0	0	10	0	0
	72	10	0	0	0	10	0	0	10	0	0	0	10	0	0
8-Methoxypsoralen	24	0	1	1	8	3	2	5	10	0	0	0	10	0	0
0.02% ethanol solution	48	0	1	1	8	4	5	1	10	0	0	0	10	0	0
(Positive control)	72	0	1	1	8	4	6	0	10	0	0	0	10	0	0

* Arbutin at a concentration of 10% in 50%, v/v, aqueous ethanol

Part 2

Hours after irradiation / Test substance	Irradiated site			Non-irradiated site		
	24	48	72	24	48	72
Arbutin *	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)
8-Methoxypsoralen 0.02% ethanol solution	10 / 10 (3.9)	10 / 10 (3.4)	10 / 10 (3.3)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)

Numerals indicate the reaction occurrence ratios.

(parenthesis) indicate the mean values of reaction.

* Arbutin at a concentration of 10% in 50%, v/v, aqueous ethanol

4. Conclusion

Phototoxicity of Arbutin was evaluated in the guinea pigs. No skin reaction was observed. Conversely, a strong phototoxicity reaction was observed with the positive control substance, 8-methoxypsoralen.

In conclusion, Arbutin has little phototoxicity potential.

Reference

1) Morikawa, F., Nakayama, Y., Fukuda, M., Hamano, M., Yokoyama, Y., Nagura, T., Ishihara, M. and Toda, K.: Techniques for Evaluation of Phototoxicity and Photoallergy in Laboratory Animals: Sunlight and Man: Fitzpatrick, T.B. et al. Ed.: University of Tokyo Press, pp. 529-557, 1974